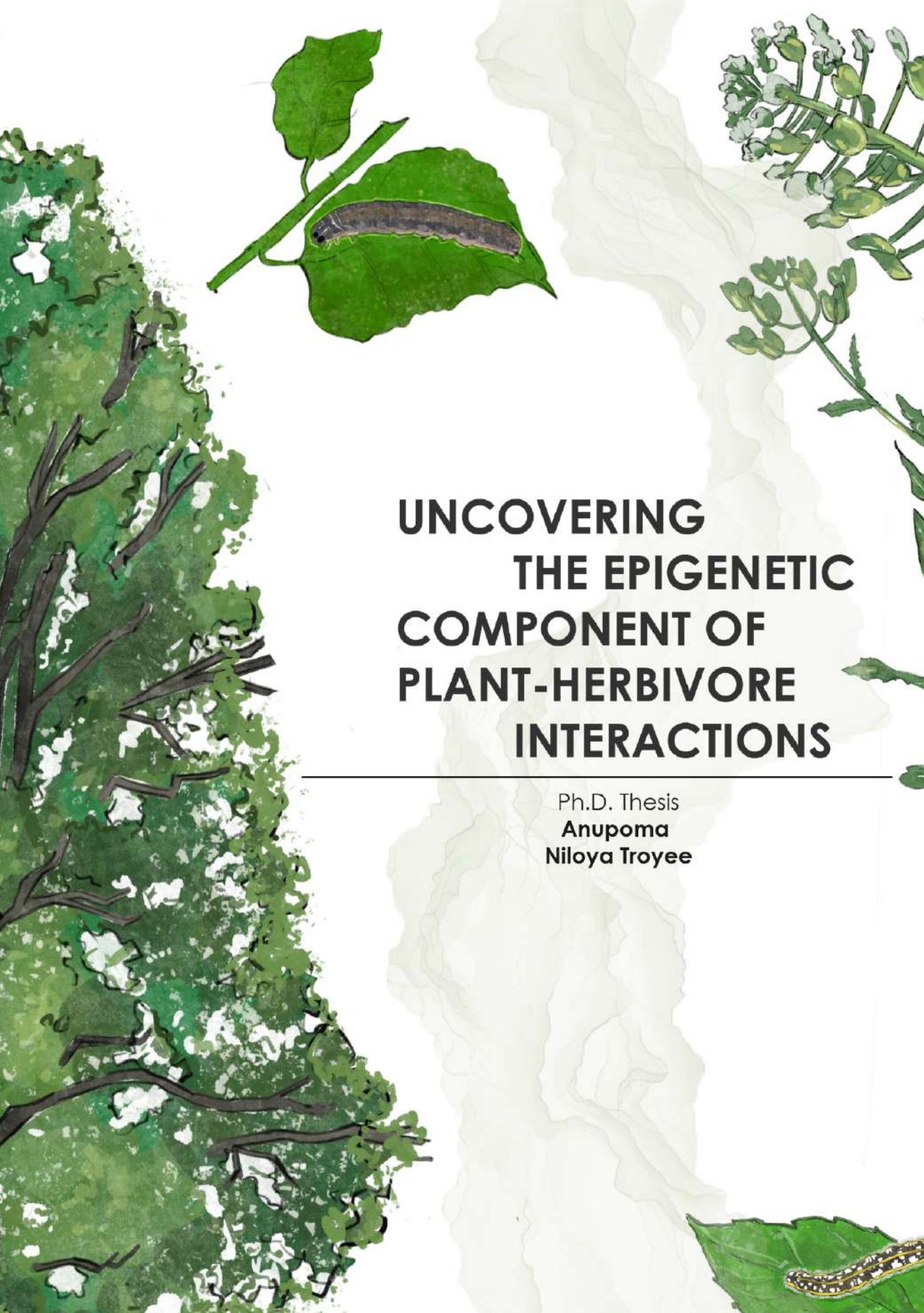

UNCOVERING THE EPIGENETIC COMPONENT OF PLANT-HERBIVORE INTERACTIONS

Ph.D. Thesis

Anupoma
Niloya Troyee

School of Doctoral Studies of Environment and Society

University of Pablo de Olavide

Biodiversity and Conservation Biology

UNCOVERING THE EPIGENETIC COMPONENT OF PLANT-HERBIVORE INTERACTIONS

Ph.D. Thesis

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Memoria presentada por la Máster en Biología de la Conservación, Anupoma Niloya Troyee, para optar al título de Doctora en la Escuela de Medio Ambiente y Sociedad, Estudios de Doctorado en Biodiversidad y Biología de la Conservación, Universidad Pablo de Olavide, Sevilla, España.

Dr. Conchita Alonso, Científica Titular, Departamento de Ecología y Evolución, Estación Biológica de Doñana (EBD-CSIC) e investigadora asociada al Programa de doctorado Medio Ambiente y Sociedad de la Universidad Pablo de Olavide y **Dr. Mónica Medrano**, Titulada Superior Actividades Técnicas y Profesionales, Departamento de Ecología y Evolución, Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (EBD-CSIC) e investigadora asociada al Programa de doctorado Medio Ambiente y Sociedad de la Universidad Pablo de Olavide.

CERTIFICAN

Que los trabajos de investigación desarrollados en la memoria de tesis doctoral titulada: "Uncovering the epigenetic component of plant-herbivore interactions" son aptos para ser presentados por la Gda. Anupoma Niloya Troyee ante el Tribunal que en su día se designe, para aspirar al Grado de Doctora por la Universidad Pablo de Olavide.

Y para que así conste, y en cumplimiento de las disposiciones legales vigentes extendemos el presente certificado a 29 de Agosto de 2024.

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Mónica Medrano

Report presented by Anupoma Niloya Troyee, to qualify for the title of Doctor of the graduate school of Environment and Society by the Pablo de Olavide, Spain

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To my parents and sister,

for supporting me unconditionally every step of the way.

"Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less."

– Marie Curie

TABLE OF CONTENT

ACKNOWLEDGEMENT

Over the past six years, I have faced countless challenges, and just as many wonderful people have helped me navigate them. I am incredibly grateful to everyone who has helped me in the process to bring this PhD thesis to life. This might be one of the most clichéd things that everyone writes, but truly, this journey has been an emotional rollercoaster, and your support has been my anchor through all the ups and downs. As I write this acknowledgment during these uncertain times, with concerns about my family's safety and the future of our country, I am reminded of how much your support has meant to me.

To my supervisors, **Conchita Alonso** and **Monica Medrano**, your guidance has been more than academic; it has given me constant inspiration and source of perseverance in my day-to-day life. I could not have asked for more supportive, kind, and helpful mentors who guided me all throughout in every phase of my thesis. From navigating bureaucratic challenges to the crucial tasks like helping me with visa issues during COVID, your support has been extraordinary. Your patience, wisdom, and care have gone beyond your roles as supervisors—you've helped me grow both professionally and personally, like family.

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And last but not the least, to Ratul, who has been with me from the very beginning of this journey: thank you for every visit, the delicious meals from

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The late planetary scientist and my favorite author Carl Sagan one saying that always mesmerized me: "Somewhere, something incredible is waiting to be known." The idea of pursuing the unknown, of learning and discovering something new, and not only gaining knowledge but also enjoying the incredible journey to that discovery, has always fascinated me. The journey might not be as exciting as the imaginary adventure in my head, but it is a real-world challenge. Yet, at the end of the day, the process of seeking out the unknown has always been rewarding, and this journey of my PhD has taught me that. This thesis is not just the result of academic work; it's a testament to the collective efforts of a community that believed in me. Thank you all, those mentioned and unmentioned, from the bottom of my heart, for being part of this transformative journey.

The thesis is based on the following papers:

List of papers

This dissertation is a cumulative work that is based on three papers that were published in scientific journals and one unpublished manuscript (Chapter 3). Two of the published papers presented a first-authorship (Chapter 2 and Chapter 4) and the other paper was published with a second-authorship (Chapter 1). Manuscripts are provided in ascending order of their appearance as chapters in this thesis:

Chapter 1 | Ramos-Cruz, D., **Troyee, A. N**., & Becker, C. (2021). Epigenetics in plant organismic interactions. *Current Opinion in Plant Biology*, *61*, 102060. https://doi.org/10.1016/j.pbi.2021.102060

Chapter 2 | **Troyee, A. N**., Medrano, M., Müller, C., & Alonso, C. (2022). Variation in DNA methylation and response to short-term herbivory in *Thlaspi arvense*. Flora, 293, 152106. https://doi.org/10.1016/j.flora.2022.152106

Chapter 3 | Medrano, M., **Troyee, A. N**., Peña-Ponton, C., Verhoeven, K. J. F., & Alonso, C. (2024). Epigenomic response to insect herbivory in Lombardy poplar: assessing geographic variance in modification of DNA methylation. unpublished manuscript.

Chapter 4 | **Troyee, A. N.**, Peña-Ponton, C., Medrano, M., Verhoeven, K. J. F., & Alonso, C. (2023). Herbivory induced methylation changes in the Lombardy poplar: A comparison of results obtained by epiGBS and WGBS. PloS One, 18(9), e0291202. https://doi.org/10.1371/journal.pone.0291202

GENERAL SUMMARY

This PhD thesis is framed within the research field of Ecological Epigenetics, focused in understanding how complex phenotypes arise from a given genotype, and how important epigenetic mechanisms and environmental factors are in shaping this process. The thesis was developed within the *EpiDiverse* European Training Network, dealing into the epigenetic contribution to plant-herbivore interactions in non-model species. The experimental part focused on two plant species widely distributed in Europe, with different life histories: *Thlaspi arvense*, often referred as annual field pennycress, and *Populus nigra* cv. 'italica' or Lombardy poplar. Structured into four chapters, the thesis begins with a comprehensive review of epigenetics' role in plant responses to diverse biotic stressors. Subsequent chapters explore the significance of changes in DNA cytosine methylation after different treatments of experimental herbivory in the two focal plant species, using three available techniques that differ in their costs (sample processing, time and price) and technical requirements (analytical tools, annotated reference genome), and including a comparison of the output produced by two Next Generation Sequencing techniques based on the analysis of DNA treated with bisulfite, the reduced representation epiGBS and the Whole Genome Bisulfite Sequencing (WGBS). The results show for the two plant species that changes in cytosine methylation have a population-specific nature and differed depending the damage is conducted by insect caterpillars (hereafter, insect herbivory) or experimentally simulated by punching the leaves and spraying jasmonic acid (hereafter, artificial herbivory). In particular, the study on *T. arvense* shows that global DNA methylation decreases after herbivory, with the magnitude of change being greater in the artificial treatment and varying with seed provenance. Additionally, it

indicates that the reduction of global DNA methylation caused by the demethylating agent 5-azacytidine applied at the seed stage, also differed in the magnitude between seed provenances. In *P. nigra* cv. 'italica', herbivory induced cytosine methylation changes mainly in the CHH context, in sequences located at intergenic regions, and with a notable overlap of these changes with transposable elements. Altogether, the studies presented within this PhD thesis provide valuable insights, although they also point to the need for further research (e.g., transcriptome analysis) to confirm the direct links between DNA methylation changes and gene expression under specific environmental conditions. The emergence of reference genome-free tools opens avenues for high-resolution plant epigenetic analysis in non-model species, demonstrating that some techniques that do not required high-quality genomic resources, such as epiGBS, can be a cost-effective and useful for characterizing genome-wide methylation changes in response to herbivory, and as here demonstrated for poplar. In conclusion, this PhD thesis significantly advances current understanding of ecological plant epigenetics, offering a new perspective and deeper molecular understanding on plant-herbivore interactions.

KEYWORDS: biotic interactions, bisulfite sequencing, DNA methylation, insect herbivory, jasmonic acid, Lombardy poplar, non-model plants, plant defense, epigenetics, *Thlaspi arvense*

Resumen General

Esta tesis se enmarca dentro de un área de conocimiento emergente que se podría denominar "Epigenética Ecológica", que esencialmente busca entender cómo en la naturaleza a partir de un único genotipo se pueden producir múltiples fenotipos, y en qué medida este fenómeno puede estar regulado por mecanismos epigenéticos y/o factores ambientales. La tesis se ha desarrollado dentro de la Red Europea de Formación *EpiDiverse,* y en ella en concreto ha contribuido a investigar el papel que juega la epigenética en las interacciones planta-herbívoro en especies no-modelo. La parte experimental de esta tesis se ha llevado a cabo usando dos plantas ampliamente distribuidas por Europa y con diferentes estrategias vitales: *Thlaspi arvese*, especie anual cuyo nombre común es carraspique, y *Populus nigra* cv. 'italica', árbol de crecimiento rápido también conocido como chopo lombardo o álamo de Italia. La tesis se ha estructurado en cuatro capítulos, comenzando con un primer capítulo de revisión bibiográfica que analiza el papel de la epigenética en las respuestas de las plantas a diferentes factores de estrés biótico. Los capítulos subsiguientes exploran los cambios producidos en la metilación de las citosinas del ADN en plantas de las dos especies de estudio sometidas a tratamiento experimental de herbivoría. Se emplearon para ello tres metodologías de análisis que difieren considerablemente en sus costes de ejecución (por el coste y el tiempo necesario para el procesado de las muestras), requerimientos técnicos (disponibilidad de herramientas analíticas y de un genoma bien anotado como referencia), incluyendo una comparación de los resultados producidos por dos técnicas de Secuenciación masiva de Nueva Generación basadas en el análisis de ADN tratado con bisulfito, de genomas reducidos (epiGBS) o genomas completos (WGBS). Los resultados obtenidos muestran que, para las dos especies de estudio, los

cambios de metilación son específicos para cada población (o procedencia) y difieren en función de si el daño es producido por insectos reales (en adelante, herbivoría de insectos) o simulado mediante perforación mecánica de las hojas con punzón y posterior rociado con una solución de ácido jasmónico (en adelante, herbivoría artificial). En concreto, en el estudio de *T. arvense* encontramos que los niveles de metilación global disminuyen tras el tratamiento de herbivoría, siendo la magnitud del cambio mayor en el caso de plantas sometidas a herbivoría artificial y variable en función de la procedencia de las semillas. Asimismo, también se demuestra que cuando se aplica el agente demetilante 5-azacitidina durante la fase de germinación de la semilla, la reducción en el nivel de metilación global es variable en su magnitud según la población de procedencia de las semillas. En *P. nigra* cv. 'italica', la herbivoría experimental produce un cambio en la metilación del ADN mayoritariamente en el contexto CHH, en secuencias que están localizadas en zonas intergénicas de su genoma y que tienen un notable solapamiento con Elementos Transponibles. En conjunto, los resultados presentados en esta tesis proporcionan valiosas y nuevas aportaciones a esta área de conocimiento, aunque también ponen de manifiesto que son necesarios estudios adicionales (p. ej., análisis de transcriptoma) para confirmar la relación directa entre los cambios de metilación del ADN y los niveles de expresión génica en determinadas condiciones ambientales. La publicación de genomas de referencia de libre acceso y el desarrollo de herramientas bioinformáticas de código abierto proporciona nuevas posibilidades para poder realizar análisis epigenéticos de alta resolución en plantas no modelo, demostrando que algunas técnicas que no requieren recursos genómicos de alta calidad, como la técnica epiGBS de representación reducida, pueden ser una opción útil y asequible para caracterizar cambios del nivel global de metilación en respuesta a herbivoría. Es importante señalar

aquí que los resultados obtenidos mediante las dos técnicas moleculares (epiGBS y WGBS) fueron congruentes. En conclusión, este trabajo de tesis doctoral ha producido un avance significativo en el conocimiento de las relaciones entre epigenética y ecología que se producen en la naturaleza en plantas no modelo, y ofrece una perspectiva innovadora al estudio de las interacciones entre las plantas y sus herbívoros.

PALABRAS CLAVE: ácido jasmónico, álamo lombardo, defensas en plantas, epigenética, herbivoría de insectos, interacciones bióticas, metilación del ADN, plantas no modelo, secuenciación bisulfito, *Thlaspi arvense*

Abbreviations

DMC:	Differentially Methylated Cytosine
DMR:	Differentially Methylated Region
NGS:	Next-Generation Sequencing
PCA:	Principal Component Analysis
PCR:	Polymerase Chain Reaction.
TE:	Transposable Element
JA:	Jasmonic Acid
BS-seq	Bisulfite sequencing
RRBS:	Reduced Representation Bisulfite Sequencing
WGBS:	Whole Genome Bisulfite Sequencing
GO	Gene Ontology

Nucleobases:

A: Adenine

C: Cytosine

G: Guanine

T: Thymine

H: Adenine, Cytosine or Thymine

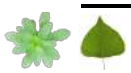
GENERAL INTRODUCTION

THEORETICAL BACKGROUND

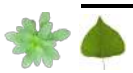
HERBIVORY: AN ESSENTIAL INTERACTION IN TERRESTRIAL ECOSYSTEMS

Herbivory, the consumption of any plant tissue by animals, is a main link in food-webs and a key ecosystem process with a large ecological impact on plant community composition, population dynamics of plants and herbivores, nutrient cycling and energy flow (Schmitz, 2008). The effect of herbivory depends on herbivore feeding type and intensity which can cause serious damage on plant primary production in both natural communities and industrial crops (Schowalter, 2022). Herbivores encompass a diverse range of organisms, such as mollusks, arthropods, and vertebrates. These organisms have developed specialised metabolism and feeding behaviours that enable them to consume plants as their primary food source. As a result, almost all plant species are subject to some degree of herbivory (Bruce, 2015). Insects are the primary herbivores in some terrestrial ecosystems, and their effect on primary production can rival or even exceed that of large vertebrates (Schowalter, 2022). For example, it has been estimated that phytophagous insects are able to consume over a fifth of all plant biomass produced annually in natural ecosystems (Turcotte et al., 2014) and are responsible for 18-26 % of annual crop losses worldwide (Culliney, 2014). Nevertheless, growing evidence indicates that effects of herbivory on ecosystem processes, including primary production, are extremely complex. Furthermore, it is possible that long-term compensatory effects to adjust over time could partially offset short-term negative effects under certain conditions (Schowalter, 2022).

The relationship between plants and insect herbivores represents one of most ancient and widespread interactions on the planet (Labandeira, 2007), it is believed to be a consequence of the conquest of dry land



(terrestrialization) by plants, and it is one of the classical examples that illustrates how coevolution does contribute to species diversity on earth (Cornell & Hawkins, 2003; Zangerl & Berenbaum, 2003). Insect herbivores are frequently categorized as either generalists or specialists, with generalists consuming plants from a variety of plant families and specialists consuming only plants that belong to the same family or genus and, thus, have more similar chemical composition (Schoonhoven et al., 2005). Plants have evolved a diverse array of defenses including physical barriers, such as leaf hardness, trichomes, or hairs, and multifaceted chemical defenses, some of which are highly dynamic and mainly induced when herbivores are detected (Howe & Jander, 2008). Predictability of damage both within and across generations will determine the adaptive potential of a constant investment in defense versus phenotypic plasticity and bet-hedging defense strategies to overcome the negative consequences of herbivory (Herman et al., 2014; Mertens et al., 2021). Defense induction is considered as a cost-saving strategy in the face of unpredictable and variable herbivory, some responses are able to discriminate mechanical damage from insect herbivory, and induction is generally regulated by plant hormones, e.g. in the case of chewing insects typically involve the activation of the jasmonic acid signaling cascade leading to prioritization for plant defense when attack is detected (Howe & Jander, 2008; Züst & Agrawal, 2017). Furthermore, inducible defenses can involve either increased secondary compounds in plant tissues as direct defense or the emission of volatile organic compounds (VOCs) that attract the natural enemies of herbivores that will reduce damage or increased insect resistance in neighboring plants serving in both cases as indirect defenses (Karban et al., 1999, 2014). Finally, it is important to consider that in the wild, individual plants usually live in heterogeneous environments with limited amounts of resources that should be allocated to growth, reproduction and defense, this



means that defense is usually costly and, thus, in addition to variation across species in the nature of defense we should also expect intraspecific quantitative variation in defense traits (Cornell & Hawkins, 2003; Endara & Coley, 2011; Karban & Baldwin, 1997; Moore et al., 2014; Verçosa et al., 2019)

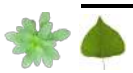
Current understanding of the molecular basis of plant defense mechanisms and their costs is improving with the development of modern molecular biology techniques and "omic" tools, and their application to a few model systems (reviewed in Erb & Reymond, 2019; Kessler & Baldwin, 2002). Yet, in non-model plants, much remains unknown about phenotypic plasticity and the regulation of gene expression that mediate defense responses. Epigenetic mechanisms have emerged as another regulatory system involved in plant defensive responses and plant memory (Colicchio et al., 2015; Herrera et al., 2019; Holeski et al., 2012; Latzel et al., 2012), although we still know relatively little about their mode of action. Thus, the main aim of this thesis is to contribute to uncover the role of epigenetics in the fascinating interaction between plants and insect herbivores, using unexplored non-model plants and manipulative approaches, as I explain in detail below.

PLANT EPIGENETICS: CONCEPT AND MECHANISTIC BASIS

The concepts of "epigenotype" as an intermediate stage between the observed phenotype and the heritable genotype, and "epigenetics" as the branch of science studying it, were first introduced by Waddington in 1942, before DNA was discovered and the complexity of gene expression was appreciated: "*We certainly need to remember that between genotype and phenotype, and connecting them to each other, there lies a whole complex of developmental processes. It is*

convenient to have a name for this complex: 'epigenotype' seems suitable" (Waddington, 2012). Coined to explain animal development, epigenetic mechanisms resulted as key to understanding the genome expansion and the proliferation of transposable elements (TEs) discovered in maize by Barbara McClintock (Fedoroff, 2012). More recently, the concept has been adapted to refer to mechanisms that induce heritable changes in gene expression regardless of nucleotide sequence variation in DNA (Fedoroff, 2012). The relevance of this field has been gradually disclosed since then and interest in plant ecology and evolution has increased in the last two decades (Akimoto et al., 2007; Bossdorf et al., 2008; Jablonka & Raz, 2009; Johannes et al., 2009; Johannes & Schmitz, 2019).

Epigenetic regulation primarily involves DNA methylation, histone tail modifications and the action of small non-coding RNAs that together alter the chromatin structure and determine changes in individual phenotypes without changing the DNA sequence (Feng, Jacobsen, et al., 2010; Pikaard & Mittelsten Scheid, 2014). The term DNA methylation refers to the addition of a methyl group to the DNA base cytosine, forming 5-methylcytosine (Pikaard & Mittelsten Scheid, 2014). Histone tail modification refers to the addition of certain chemical marks (methylation, acetylation, ubiquitination, phosphorylation, biotinylation, and ADP-ribosylation) that can be covalently introduced at different positions (mostly lysine and arginine residues) of the histone proteins (H2A, H2B, H3, and H4) involved in DNA structure and compaction (Sadakierska-Chudy & Filip, 2015; Foroozani et al., 2022). Lastly, small RNAs are a suite of noncoding RNAs with variable length (mainly 21–24 nucleotides) that can bind to certain complementary DNA sequences and suppress sequence-specific gene expression (Simon & Meyers, 2011). In particular, 24-nt small interfering RNA (siRNA) can lead to de novo DNA



methylation in the three contexts through a pathway called RNA-directed DNA methylation (RdDM) (Erdmann & Picard, 2020). This pathway is also self-reinforcing, which could further contribute to transgenerational transmission of DNA methylation (Erdmann & Picard, 2020).

DNA methylation plays a crucial role in regulating genome stability, transposon silencing, cell identity, and responses to environmental stress in plants (Feng, Cokus, et al., 2010; Pikaard & Mittelsten Scheid, 2014). In plants, it occurs in three sequence contexts (CG, CHG, and CHH; where C = Cytosine, G = Guanine and H = Adenosine / Cytosine / Thymine). CG and CHG methylation are symmetrical (they occur on both complementary DNA strands), while CHH methylation is asymmetrical (Cao & Jacobsen, 2002). Current molecular understanding of plant epigenetic machinery has been gained mainly by analyzing the model plant *Arabidopsis thaliana*, a short-lived annual plant with a small genome and outstanding low DNA methylation (Alonso et al., 2015). Methylation in CG is maintained by the MET1 methyltransferase, which uses hemimethylated DNA as a substrate and recruits the VARIANT IN METHYLATION (VIM) protein family of SET- and RING-associated domain proteins to the replication complex (Feng, Cokus, et al., 2010; Saze et al., 2003; Zhang et al., 2018). The chromatin protein DECREASED DNA METHYLATION 1 (DDM1) is also important in maintaining CG methylation (Jeddeloh et al., 1999). Moreover, chromomethylases are DNA methyltransferases characteristic of plants, CHROMOMETHYLASE 3 (CMT3) is responsible for maintaining CHG methylation, while DRM2 or CMT2, depending on the genome location, is responsible for maintaining CHH methylation (Stroud et al., 2014; Zhang et al., 2018). In particular, DRM2 maintains CHH methylation at RdDM target regions, which are typically found at the margins of transposons,

heterochromatin, evolutionarily immature transposons, and other repetitive sequences in euchromatic chromosomal arms (Matzke & Mosher, 2014; Zhang et al., 2018).

I will end up this section highlighting the huge interspecific diversity of plant epigenomic features (Springer et al., 2016). Genome-wide DNA methylation, estimated as the percentage of all genome cytosines that are methylated, ranges approximately between 5 and 45% in different samples of Angiosperms and shows correlated evolution with genome size (Alonso et al., 2015; Springer et al., 2016). Further, CG methylation in the transcribed regions of genes, i.e., gene body methylation, ranges between 2 and 86% in the group of Viridiplantae analyzed (Bewick & Schmitz, 2017). DNA methylation exhibits a strong phylogenetic signal (Alonso et al., 2015, 2019; Bewick & Schmitz, 2017), and some interesting biogeographic divergence (Alonso et al., 2019) suggesting that epigenetic responses can largely differ across species in a way shaped by long-term evolution. Noteworthy, the model species *A. thaliana* has a very small-genome and almost the lowest methylation level recorded up today (Alonso et al., 2019). Thus, transferring the methods developed and the knowledge gained by investigating DNA methylation in *A. thaliana* to other non-model species is challenging and timely to move forwards the field of Plant Epigenetics. This thesis aims to contribute to this goal and, in particular, the last chapter is devoted to compare the results of applying two single-base resolution methods to evaluate methylation changes induced by insect herbivory in the Lombardy poplar (see below).

ECOLOGICAL RELEVANCE OF EPIGENETIC REGULATION IN PLANTS

DNA methylation is highly dynamic and the diversity of methylation variants is usually significantly larger than the diversity of genetic variants in wild plant populations (see e.g., Medrano et al., 2020, and references therein). Changes in methylation, frequently denoted as epimutations, can occur randomly or in response to environmental factors (Johannes & Schmitz, 2019). Epimutations have other two characteristics that make them particularly interesting to understand plant adaptation, they are much more frequent than genetic mutations and they are transmissible to some extent across generations but also reversible (Johannes & Schmitz, 2019). Together with the potential impact of environmental conditions for triggering above mentioned effects, epimutations could lead to intraspecific geographic variation in DNA methylation (see e.g., Galanti et al., 2022; Kawakatsu et al., 2016).

In order to play a significant role in the adaptive evolution of plant populations, epimutations must affect the phenotype, most likely via gene expression changes, and be at least partially independent of the genome sequence variation. First, environment-triggered epimutations could transmit responses to changes in environmental conditions within generations, helping to explain phenotypic plasticity and priming (Herman et al., 2014). At the same time, this process can reduce or enhance offspring fitness depending on parental stress leading to transgenerational phenotypic plasticity that would be selected for depending on the rate of environmental fluctuation (Herman et al., 2014). In addition, heritable epigenetic variants that arise stochastically can increase phenotypic variation and may be under natural selection contributing to plant adaptation and population differentiation, independently from DNA sequence variation (Herrera et al., 2016 and

references therein), and may be used to track recent evolutionary events on a scale of years to centuries (Yao et al., 2023). It is also necessary to highlight that if the expression of environment-triggered epimutations varies between plant populations which remains largely unknown. Finally, moving to the smallest hierarchical scale, substantial methylation changes have been also recorded across modules of plant individuals (Herrera et al., 2022). Interestingly, module methylation correlated with fitness-related traits and more importantly the offspring seedlings resemble their maternal-module methylation characteristics (Herrera et al., 2022).

Altogether, epigenetic variation offers an extra layer of variation between the genetic features of plant individuals, and the environmental features of the place in which they live, providing opportunities for phenotypic variation that could foster adaptation in these sessile and modular organisms that constitute a key element in all ecosystems. This thesis explores phenotypic and epigenetic variation elicited in response to insect herbivory and the extent to which such response could vary geographically. The evolutionary potential of sub-individual epigenetic variation has been very recently uncovered and its analysis is out of the scope of this thesis, although it was controlled when sampling leaf materials for DNA methylation analysis.

EVIDENCE OF EPIGENETIC REGULATION IN PLANT DEFENSE

Epigenetic mechanisms are important regulators of gene expression and have been involved in plant biotic interactions including plant-herbivore interactions (Ramos-Cruz et al., 2021). Many plants are able to induce or rapidly activate specific defenses after an attack is perceived, which can minimize the damage caused by herbivores, including short-time production of secondary metabolites and proteins that have toxic, repellent, and/or anti-

nutritional effects on the herbivores (Züst & Agrawal, 2017) and increased trichome densities (Colicchio et al., 2015). In some cases, plants can also be primed to respond more efficiently against future attack via faster and stronger gene activation (Mauch-Mani et al., 2017; Rasmann et al., 2012) and they can even pass signatures of attack to the next generation, thus rendering the progeny more resistant against subsequent herbivory (Akkerman et al., 2016; Holeski et al., 2012). For instance, a combination of phytohormone signaling, small RNA-mediated gene silencing and DNA methylation seem to play a central role in regulating transgenerational plant defense in *A. thaliana* (Baldwin, 1998; Holeski et al., 2012; Rasmann et al., 2012; Züst & Agrawal, 2017), whereas both DNA methylation and small RNAs are involved in *Mimulus guttatus* transgenerational defense induction (Colicchio et al., 2015, 2018; Colicchio & Herman, 2020). To corroborate the relevance of epigenetic variation involved in plant defensive responses more studies of wild plants are required, including the analysis of natural populations with contrasting environmental conditions.

A combination of manipulation of DNA cytosine methylation with controlled herbivory experiments can be a fruitful strategy to uncover the epigenetic contribution to specific plant responses after herbivory (Herrera et al., 2019; Latzel et al., 2020). The use of inhibitors of the activity of DNA methyltransferase enzymes such as 5-azacytidine, 5-aza-2'-deoxycytidine or zebularine provides a tractable way to modify DNA cytosine methylation in plants, which could enlarge natural variation in this particular epigenetic feature, while controlling for relatedness (Alonso et al., 2017; Puy et al., 2018). Application of these compounds at seed stage can influence on plant phenotypes, including flowering phenology (Alonso et al., 2017; Fieldes & Amyot, 2000; Kondo et al., 2006) and might at least partially erase memories

of past stress (see e.g., Akkerman et al., 2016). 5-azacytidine is a structural analogue of 5-methyl-cytosine that can be incorporated into DNA where it establishes a covalent bond with a methyltransferase enzyme that cannot be easily reversed. The enzyme is trapped reducing the number of active DNA methyltransferase enzymes in the cells and therefore passively inducing DNA demethylation (Griffin et al., 2016; Lopez et al., 2016). Current evidence supports that application of 5-azacytidine at seed stage leads to an overall reduction of DNA methylation in flowering individuals of *Linum usitatissimum* (Fieldes & Amyot, 2000), *Thlaspi arvense* (Troyee et al., 2022) and *Erodium cicutarium* (Balao et al., 2023), supporting the value of this methodology (used in Chapter 2) to investigate epigenetic regulation that could be transmitted to offspring.

MATERIALS AND METHODS

STUDY SYSTEMS

This thesis was developed within the *EpiDiverse* - Marie-Slowodska Curie European Training Network (www.epidiverse.eu) and therefore the initial focal systems were the three plant species selected by this network: the annual herb *Thlaspi arvense* (Brassicaceae), the perennial herb *Fragaria vesca* (Rosaceae) and the woody deciduous tree *Populus nigra* var. *'italica'* (Salicaceae). They were considered appropriate focal species to linking ecology, molecular biology and bioinformatics in fostering plant epigenetic research because (a) they are common and widely distributed across Europe; (b) they have manageable genome sizes; (c) a good quality reference genome already existed —or was generated by the *Epidiverse* network based on available draft genomes in parallel to projected experiments—; and (d) they represented different plant

life-histories and ecological characteristics. Unfortunately, my studies on *F. vesca* had technical problems for obtaining good quality sequencing that could not be solved on time and have been thus excluded.

Figure 1. ***Focal study plants in their natural habitat.*** **a)** *Thlaspi arvense* (Brassicaceae) is a foetid, hairless annual plant with upright branches and arrow-shaped, narrow, toothed stem leaves. It flowers early in the spring, producing small white flowers in racemes, each with four sepals and four petals, and yields heart-shaped, flat seed pods containing up to 14 small brown-black seeds. **b)** *Populus nigra* **var. '*italica*' (**Salicaceae) is a fast-growing tree with dark gray, deeply fissured bark on mature individuals. It features diamond-shaped, shiny deciduous leaves with serrated edges that turn yellow in the fall. This dioecious species flowers early in spring and has male and female flowers on separate trees. Mature trees grow up to 30 m and can live for > 200 years. The Lombardy clone has been artificially propagated all over Europe, being suitable for windbreaks and public gardens.

System 1: *Thlaspi arvense* (field pennycress), is a weedy annual species with a short life cycle, that belongs to Brassicaceae family (Figure 1a). This pennycress is diploid (2n = 14) and has a small genome size (~500 Mb). It is distributed worldwide and has a high degree of ecotypic variation, including differences in its life-cycle phenology and the need of vernalization for flowering induction (Burn et al., 1993; Sedbrook et al., 2014). The European

populations exhibited moderate genetic variation and epigenetic differences that are linked to environmental drivers (Galanti et al., 2022). Natural herbivores of Brassicaceae species are mostly specialist herbivores including larvae of Lepidoptera (e.g., *Trichoplusia*, *Plutella*, *Pieris*) and some sap-feeding aphids (Lowenstein & Minor, 2018). We selected the cabbage moth, *Pieris brassicae* (L.) (Lepidoptera: Pieridae), with a broad distribution range, which covers from North Africa all across Europe and Asia, and it is specialized in feeding on Brassicaceae (Metspalu et al., 2003) **Chapter 2**).

System 2: *Populus nigra* (black poplar) is a diploid and fast-growing deciduous tree of Salicaceae family (Figure 1b) native to Eurasia and with a compact genome size (~500 Mb). It is an ecologically important, sexually reproducing pioneer species in riverine areas. Poplars can also propagate vegetatively, being widely used in tree biology research and grown as cultivars for commercial purposes. We focused on the Lombardy clone (*Populus nigra* var. '*italica*' Duroi; *P. nigra* hereafter), which has been planted massively all over Europe since the 19th century, because we aimed to reduce genetic variability across samples to better uncover epigenetic mechanisms. Among potential herbivores, we selected the larvae of the gypsy moth *Lymantria dispar* (L.) (Lepidoptera: Erebidae), with Eurasian origin and a broad distribution range which covers Europe, Africa, and North America (Boukouvala et al., 2022). Caterpillars of *L. dispar* are highly polyphagous including several trees and shrubs and they have been used as model system to study plant induced defences and their effect on parasitoid development (**Chapters 3 & 4**). In particular, in **Chapter 3** we assessed whether methylation changes in response to herbivory vary across poplars originated from distant geographic provenances. **Chapter 4** takes advantage of the clonal origin of Lombardy poplar (with *a priori* homogenous genetic background) to compare the outputs

of two molecular techniques used for the analysis of methylation changes based on bisulfite conversion and deep sequencing of DNA that could be useful in ecological studies.

PLANT AND INSECT MATERIALS

As mentioned above, in this thesis, individuals from natural populations of field pennycress and Lombardy poplar at distant geographic locations within Europe were used to evaluate potential geographic variation in the response to herbivory (Chapters 2 & 3, respectively). **Table 1** summarizes the geographic location and plant materials collected in collaboration with partners of the *EpiDiverse* network that were used for experimental studies: *T. arvense* seeds - University of Tübingen, Germany, and *P. nigra* cuttings - Philipps-Universität Marburg, Germany and NIOO-KNAW Wageningen, Netherlands.

Second instar larvae of *Lymantria dispar* were provided by Dr. Sybille Unsicker's lab (Max Planck Institute for Chemical Ecology, Jena, Germany). Eggs and second instar larvae of *Pieris brassicae* were obtained from a commercial supplier (www.lombricesdecalifornia.com)

Table 1. Summary of the geographic locations, collected plant materials, study design (including final sample size), technique applied and methylation estimate obtained to quantify changes in DNA methylation after insect and artificial herbivory in the three experimental studies of this thesis (Chapters 2 to 4).

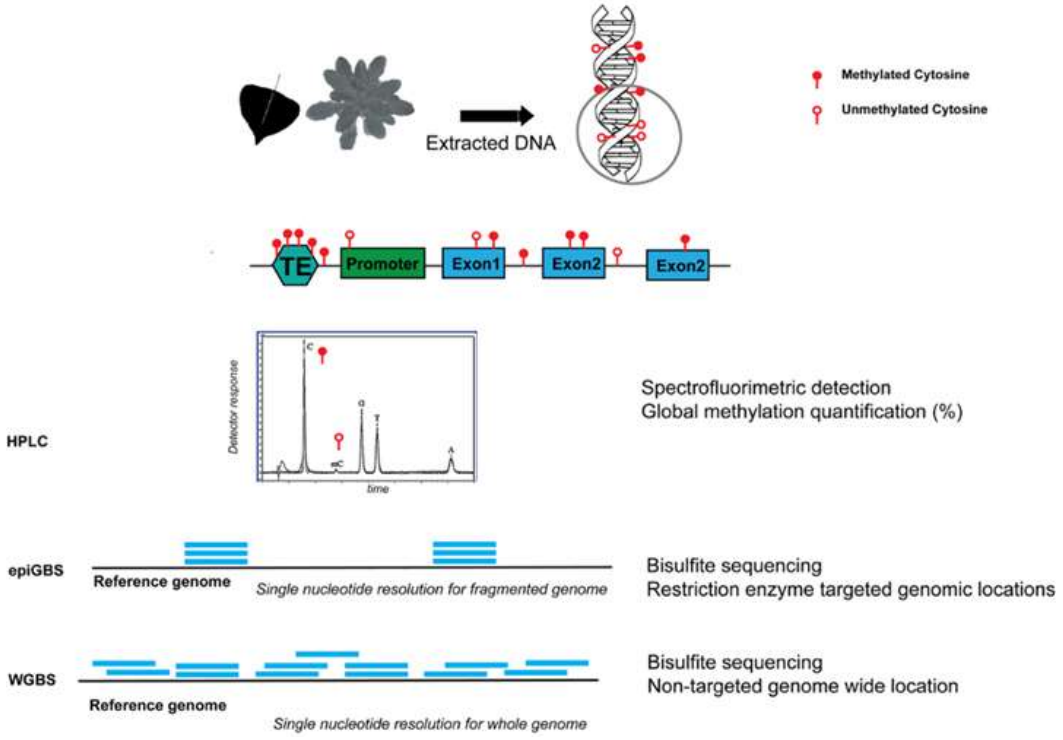
Plant species (Chapter)	Geographic location	Plant material	Study design (sample size)	Technique applied / Methylation estimate
Thlaspi arvense (Chapter 2)	North Germany, Central Sweden	Seeds	2 populations * 2-3 mothers * 3 herbivory levels * 2 demethylation levels * 3-4 seedlings (N = 113)	HPLC / Global methylation changes
Populus nigra var. '*italica*' (Chapter 3)	Spain, Italy, Poland	Cuttings	3 populations * 2-3 ortets * 3 herbivory levels * 3 ramets (N = 63)	epiGBS / Global and genome site-specific changes
Populus nigra var. '*italica*' (Chapter 4)	Italy	Cuttings	1 population * 3 ortets * 3 herbivory levels * 3 ramets (N = 27)	epiGBS + WGBS Global and genome site-specific changes

EXPERIMENTAL DESIGN AND ANALYTICAL TECHNIQUES

As mentioned above, different plant species can use different strategies in response to herbivory and the induction of specific defenses can be tailored according to the magnitude and frequency of damage, and the identity of herbivore species (Howe & Jander, 2008; Züst & Agrawal, 2017). In particular, mechanical wounding and insect herbivory are known to induce distinct responses in some plant species, and jasmonic acid (and related compounds) is a key regulator of the response (Howe & Jander, 2008; Rasmann et al., 2012; Züst & Agrawal, 2017). Accordingly, in the three experimental studies of this thesis (**Chapters 2-4**) plants were assigned to one of three herbivory treatments (i) Insect herbivory: defoliation by larvae of lepidopteran species, (ii) Artificial herbivory: punching holes and spraying a solution of jasmonic acid (1mM) and (iii) Control undamaged plants that were sprayed with a similar amount of water. Both insect and artificial herbivory treatments were performed twice in order to induce a priming effect (first treatment) and elicit a stronger and/or quicker response during the second treatment (Mauch-Mani et al., 2017). We were interested in characterizing systemic changes that occur at not only the local damaged tissue but spread within plants challenged by herbivory because systemic responses are more likely to have a priming effect within- and across-generations (Howe & Jander, 2008; Züst & Agrawal, 2017). Thus, leaf material for DNA methylation analyses was collected from undamaged and completely expanded leaves 24 h after the second event and immediately frozen.

After selection of the study systems and experimental treatments, we ought to decide how to quantify changes in DNA methylation after herbivory according to the pursued accuracy and available resources. Main characteristics of the three methods used in this thesis are explained in detail

in **Box 1**. In Chapter 2, a high-performance liquid chromatography (HPLC) was used to estimate changes in global DNA methylation level. In Chapter 3, context specific changes and their genomic location together with global methylation were estimated by a reduced representation bisulphite sequencing technique called epiGBS (Gawehns et al., 2022). In Chapter 4, both epiGBS and Whole Genome Bisulphite Sequencing (WGBS; Suzuki et al., 2018) were applied to the same DNA samples in order to compare the outputs of these two molecular techniques.



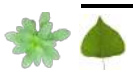
Box 1. Methods applied in this thesis for studying changes in DNA methylation after insect and artificial herbivory. *HPLC*: High-Performance Liquid Chromatography estimates the total amount of cytosines (C) and 5-methyl cytosines (5mC) in a small amount of genomic DNA (Alonso et al., 2016); *epiGBS*: epi-Genotyping By Sequencing evaluates methylation at single base resolution in a reduced portion of the genome, applying bisulfite sequencing to enzymatically digested genomic DNA (Gawehns et al., 2022); and *WGBS*: Whole Genome Bisulfite Sequencing evaluates methylation at single base resolution applying bisulfite sequencing to genomic DNA mechanically fragmented and comparing the output to a high-

quality reference genome (Suzuki et al., 2018). Below we summarize the characteristics of wet-lab protocol, target genomes and output coverage, and their respective technical requirements.

(1) Protocol: HPLC is a chromatographic technique that quantifies DNA methylation levels of fully digested DNA, using a liquid mobile phase and a solid stationary phase suitable to separate C from 5mC. Concentration of the two nucleotides is obtained and their relative proportion estimated to provide an overall global methylation estimate (%). For the two bisulfite sequencing techniques (epiGBS and WGBS), DNA is chemically treated with sodium bisulfite, which converts C into uracil. DNA strands are then PCR amplified, converting the uracils into thymines (T). Methylated cytosines (5mC), on the other hand, remain unconverted. Next generation sequencing of the converted DNA provides information on the methylation level of individual nucleotides by comparison of C/T proportions of the converted samples analyzed to the non-converted sequence of good quality reference genome. epiGBS includes a restriction enzyme digestion of genomic DNA as a first step to reduce the portion of genome to be sequenced. Finally, WGBS targets analyzing DNA methylation across the complete genome and requires a high quality reference genome for bisulfite mapping, and genome-wide methylation calling. Bioinformatic analyses of epiGBS and WGBS data are key to perform quality control of sequence data, adapter trimming, SNP calling and methylation estimates. The epiGBS2 tool (Gawehns et al., 2022) describes a pipeline that performs all those steps for species with or without a reference genome. A consensus protocol for analyses of differential methylation between groups of samples is not yet available. The epiDiverse toolkit (Nunn et al., 2021) provides bioinformatic pipelines for mapping, the calling of methylation values and differential methylation between groups, epigenome wide association studies, and a novel implementation for both variant calling and discriminating between genetic and epigenetic variation.

(2) *Target genomes and coverage*: HPLC does not require any previous genomic information of the target genome and it can be applied to species with large and polyploidy genomes. It provides an accurate estimate of genome-wide global DNA cytosine methylation level per sample but it does not provide cytosine context specific information. Library preparation for bisulfite sequencing requires previous knowledge of genome size, the design of custom adapters and methylated barcodes suitable to analyze several samples per sequencing line (to reduce costs), and most of the analyses developed to estimate genetic variants and methylation estimates presume diploid genomes. In particular, epiGBS provides the methylation status of specific DNA fragments targeted by using double restriction enzymes while WGBS analyzes non-targeted fragments, which theoretically cover the entire genome and thus provide an unbiased view of DNA methylation landscape of organisms (Suzuki et al., 2018). The two methods provide methylation estimates of C in all sequencing contexts with a higher coverage per position and price obtained by epiGBS (i.e., the obtained coverage will vary with genome size and budget available).

(3)*Technical requirements*: Development of the HPLC technique requires an HPLC system equipped with fluorescence detector, and trained personnel to obtain accurate estimates. Its general maintenance is moderate to intensive and data analyses can be conducted in a personal computer. Library preparation for epiGBS should be conducted at home and it requires a well-equipped lab and trained personnel able to produce equimolar representation of multiplexed samples. After an initial investment for designing appropriate adapters and methylated barcodes, it can be a more cost-effective technology than WGBS because it targets a section of the genome and thus a larger number of samples can be sequenced per line. Library preparation for WGBS can be ordered to commercial sequencing services. The two methods further demand bioinformatic infrastructure and training due to its data-intensive nature. WGBS incurs higher costs compared to alternative techniques, especially when dealing with large genome species and it is mainly worth if a reference genome with good quality annotation of genes, transposable elements and repetitive regions is available for accurate functional annotation.



OBJECTIVES AND THESIS STRUCTURE

This doctoral thesis aims to contribute to gain generality in current understanding about the epigenetic contribution to plant defense in response to insect herbivory. The general hypothesis is that plant-herbivore interactions can cause epigenetic modifications in plants, and these will contribute to induced plant defense. This hypothesis was examined by reviewing evidence available in scientific publications and conducting experimental research with four particular objectives:

O1. - Characterizing DNA methylation variation associated with insect herbivory in natural plant populations;

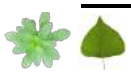
O2. - Analyzing the effect of artificially altered DNA methylation induced by chemical methyltransferase inhibitors (5-Azacytidine) on plant responses;

O3. - To investigate the changes in phenotypic traits underpinned by herbivory induction and its correlation with epigenetic variation;

O4. - Comparing the outputs that different methods provide to offer reliable insight about methylation changes in DNA in response to insect herbivory.

The results obtained are presented in four chapters:

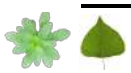
Chapter 1 ("**Epigenetics in plant organismic interactions**"), published in *Current Opinion in Plant Biology vol 61,* provided an overview of the suite of plant epigenetic regulatory processes involved in either beneficial interactions or defense against pathogens and herbivores. We conducted a literature review published until May 2021 focused on recent studies analyzing DNA



methylation, histone modifications, and small RNAs that contribute to the intricate regulatory mechanisms able to adjust molecular processes necessary for beneficial biotic interactions and defense against pest, pathogens and herbivores. The review was organized according to the type of epigenetic modification rather than by organism. The contribution of epigenetic regulation in plant defense, symbiosis, and parasitism is discussed. This publication addresses O1.

Chapter 2 ("**Variation in DNA methylation and response to short-term herbivory in *Thlaspi arvense***"), published in *Flora vol 293*, showed that experimental seed demethylation with 5-azacytidine altered global methylation in leaf DNA of early-flowering adult plants. Global DNA methylation was analyzed by HPLC, a highly precise chromatographic technique (Alonso et al., 2016). Furthermore, we assessed phenotypic and defense traits in response to demethylation and herbivory treatments, including concentration of leaf glucosinolates, final stem biomass, fruit and seed production, and seed size on *T. arvense* plants that came from two European populations with contrasting flowering phenotypes expected to differ in the response to experimental demethylation. We found that early- and late-flowering European ecotypes responded differently to the two treatments, suggesting that plant genetic background and timing of damage can affect herbivory-induced phenotypic plasticity. This publication addresses O1, O2 and O3.

Chapter 3 ("**Epigenomic response to insect herbivory in Lombardy poplar: assessing geographic variance in modification of DNA methylation**"), unpublished manuscript, assessed the heterogeneity in the response to herbivory of clonal individuals of the Lombardy poplar obtained from three distant European populations, conducted under controlled



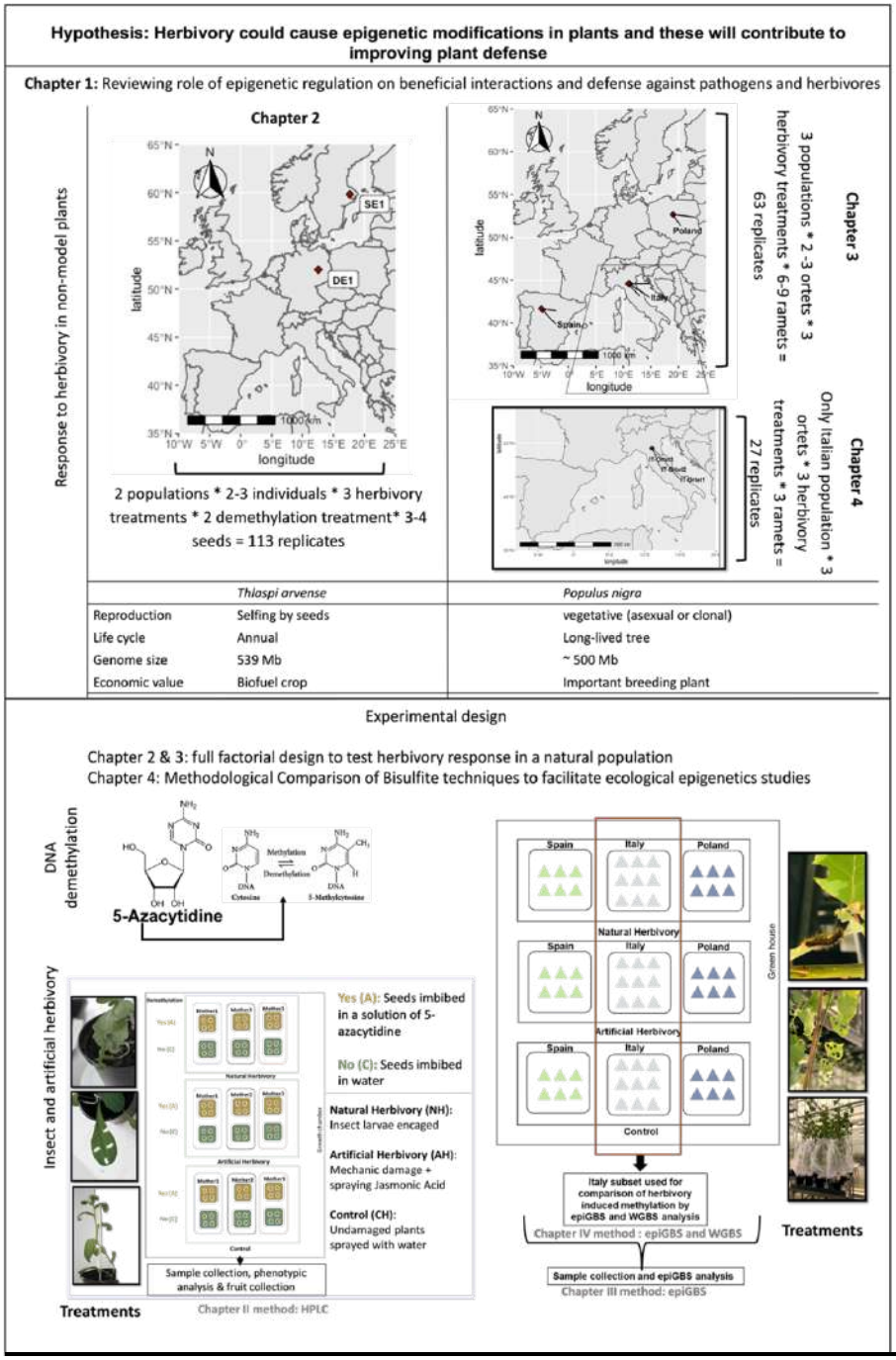
experimental conditions. We used epiGBS, a high-resolution approach for quantifying DNA methylation at single nucleotide level, to evaluate context-specific responses to insect and artificial herbivory. We found that herbivory significantly increased methylation in CHH with a stronger effect of insect herbivory. Less than 30 % of Differentially Methylated Cytosines (DMCs) were shared between the two herbivory treatments suggesting that the effects of insect consumption were not perfectly mimicked by mechanical damage and JA-spraying. Furthermore, the response obtained was not homogenous across the three populations studied. We found differences in the magnitude of the response because Polish plants had the highest number of DMCs induced by artificial herbivory while Spanish plants responded more to insect herbivory, and in the specific loci whose methylation was altered, because DMCs induced by any of the two treatments were rarely shared across the study populations. Across all populations and contexts, most of the "*strongly responding*" DMCs showed greater densities within gene bodies. Altogether, our results suggest that epigenetic response to herbivory can vary according to plant origin, likely associated to long-term exposure to contrasting climates and other environment relevant features, even in systems that lack genetic variation. This unpublished manuscript addresses O1.

Chapter 4 ("**Herbivory induced methylation changes in the Lombardy poplar: a comparison of results obtained by epiGBS and WGBS**"), manuscript published in *PLOS One vol 18*, compared and validated suitability of epiGBS technique for analyzing plant response to the specific stress induced by insect and artificial herbivory in comparison with the gold standard (but significantly more expensive technique) which interrogates methylation in Whole Genomes by Bisulphite Sequencing (WGBS). We used a small number of poplar clones with limited genetic variation and found that

the two techniques supported the specificity of the differential methylation elicited by insect and artificial herbivory at interrogated loci, and, overall, a higher frequency of hypo-methylation in the CG context and hyper-methylation in the CHH context. Thus, epiGBS succeeded to characterize global, genome-wide methylation changes in response to herbivory in the Lombardy poplar. We concluded this technique may be particularly useful for investigating stress response in non-model plants. This publication addresses O1 and O4.

GRAPHICAL ABSTRACT

Figure 2. Graphical outline summarizing the study systems and experimental design of the studies conducted to address the general objective of this thesis.



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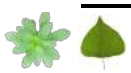

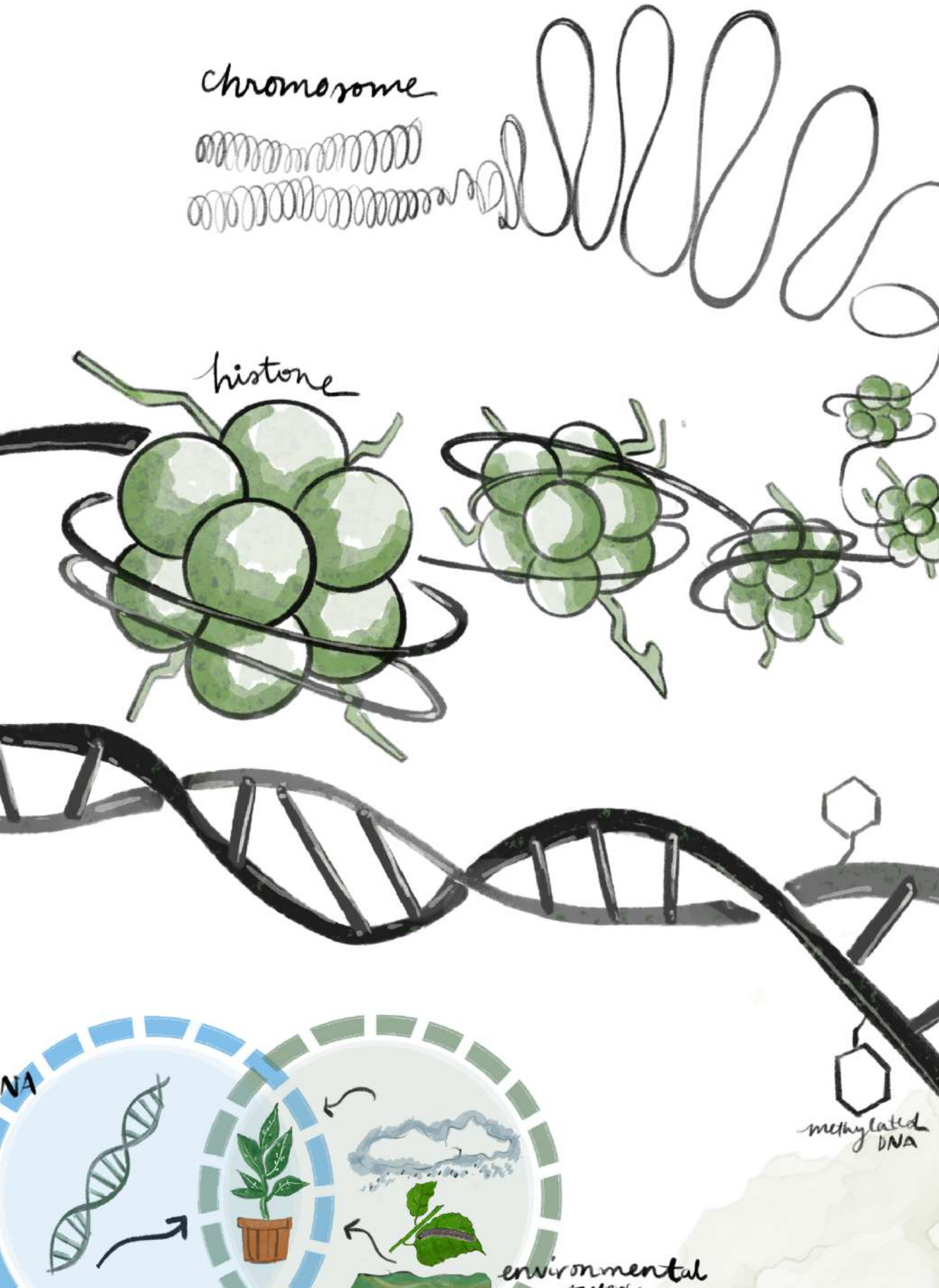
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CHAPTER 1: EPIGENETICS IN PLANT ORGANISMIC INTERACTIONS

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ABSTRACT

Plants are hubs of organismic interactions. They constantly engage in beneficial or competitive interactions with fungi, oomycetes, bacteria, insects, nematodes and other plants. To adjust the molecular processes necessary for the establishment and maintenance of beneficial interactions and for the defense against pathogens and herbivores, plants have evolved intricate regulatory mechanisms. Besides the canonical plant immune system that acts as the primary defense, epigenetic mechanisms have started to emerge as another regulatory entity and as a target of pathogens trying to overcome the plant's defenses. In this review, we highlight recent advances in understanding the contribution of various epigenetic components and of epigenetic diversity to plant-organismic interactions.

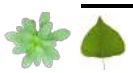
INTRODUCTION

Plants constantly face pressure from their biotic environment and have evolved multiple layers of defense. Thorns, spikes, and cuticular barriers are the most obvious physical hurdles that herbivores and pathogens need to overcome. Beyond those, they often face a highly complex and effective chemical arsenal of plant specialized (aka secondary) metabolites, which are designed to deter animals, fungi, oomycetes, bacteria, or other plants (Hartmann, 2007). Finally, at the molecular level, plants have developed a broad recognition repertoire to compensate for the lack of an adaptive immune system. Plant immunity mainly relies on the perception of pathogen-associated molecular patterns (PAMPs) or of microbial effector proteins. PAMP-triggered immunity (PTI) involves diverse responses such as stomata closure, production of reactive oxygen species (ROS) and nitric oxide (NO),

biosynthesis of antimicrobial compounds, and hormonal defense responses involving salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Bigeard et al., 2015). Some pathogens can suppress PTI through the production of effector proteins that translocate into the plant cell; in a second line of plant defense, such effectors are recognized by R (resistance) proteins, leading to effector-triggered immunity (ETI), which induces programmed cell death to prevent the intruder from spreading throughout the plant tissue (Cui et al., 2015). The signaling cascades of PTI and ETI converge downstream in activating common immunity-related genes (Peng et al., 2018).

At the same time, plants need to engage in beneficial cross-kingdom interactions, e.g. with fungi and bacteria, to ensure nutrient uptake and exchange (Finkel et al., 2017). As a result, diverse strategies evolved to distinguish friend from foe and to ensure survival and fitness in changing biotic environments (Holeski et al., 2012). The proper distinction from wanted and unwanted interactions requires a fine-tuned adjustment of the regulatory systems.

While the relevance of host genotype for biotic interactions has been known for a long time, recent studies have highlighted that the plant *epi*genotype - the epigenetic configuration of the host genome at one or several genomic loci - is also part of the equation (Alonso et al., 2018). In turn, there is accumulating evidence that the plant epigenetic machinery is directly involved in plant defense responses (Deleris et al., 2016) and in the establishment of memory to environmental stress (reviewed in He & Li, 2018; Ashapkin et al., 2020). Therefore, both the epigenetic configuration of the host and the effect of biotic interactions thereon play a role in such responses. The epigenetic makeup of plants (and of most eukaryotic organisms) consists of the totality of DNA methylation, histone modifications, and - depending on the

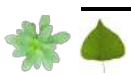


definition - small RNAs (sRNAs). Alone or in combination, these epigenetic marks define local chromatin accessibility, which ultimately determines gene expression and hence also potentially contributes to plant defence, establishment of symbiosis, etc.

In this review, we revisit the recent literature on the role of epigenetics in the establishment and modulation of plant biotic interactions. Readers interested in the epigenetic contribution to antiviral defense are referred to two recent reviews on the topic (Prasad et al., 2019; C. Wang et al., 2019). Because the interaction systems studied so far have been very diverse, we decided to structure our review by the type of epigenetic modification rather than by organism. Towards the end, we will elaborate on what we think are future questions worth addressing in the field.

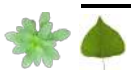
DNA METHYLATION IN INTERACTIONS WITH PESTS

Among the epigenetic mechanisms that control gene expression in eukaryotes, the one studied most extensively is DNA methylation (Feng et al., 2011; Vanyushin & Ashapkin, 2011). Two types of DNA methylation have been detected in plant genomes: rare N6-adenosine methylation (6mA) (Liang et al., 2018) and 5-cytosine methylation (5mC). Here, we will use the term DNA methylation interchangeably with 5mC. In plants, DNA methylation occurs in the symmetric contexts CG and CHG, and the asymmetric context CHH (where H is any base but G). CG methylation is the type mainly found in protein-coding genes, while repetitive sequences and transposable elements are generally densely methylated in all contexts (Cokus et al., 2008; Lister et al., 2008; López Sánchez et al., 2016). Symmetric CG and CHG DNA methylation is maintained by the DNA methyltransferase MET1 and the plant specific methyltransferase CHROMOMETHYLASE 3 (CMT3),



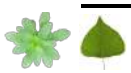
respectively, which also functionally links DNA methylation and demethylation of lysin 9 of histone H3 (H3K9me2)(Du et al., 2012; Woo et al., 2008). *De novo* DNA methylation in all contexts is controlled by the RNA-directed DNA methylation (RdDM) pathway, involving DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) (Cao & Jacobsen, 2002). Alternatively, CHH methylation is under the control of CMT2 in cross-talk with histone H3 methylation (Stroud et al., 2014). Removal of DNA methylation is carried out by DNA glycosylases of the DEMETER family via base excision (Gong et al., 2002). In *A. thaliana*, this family comprises *DME*, *REPRESSOR OF SILENCING 1* (*ROS1*), and *DEMETER-LIKE 1* (*DML1*) and *DML2* (Gong et al., 2002). DNA methylation can change dynamically in response to environmental cues, including abiotic and biotic stresses (H. Zhang et al., 2018). Moreover, because DNA methylation patterns can be copied from mother to daughter cells, it has been postulated as the carrier of heritable epigenetic information.

The role of DNA methylation in plant defense has been extensively characterized (Hewezi et al., 2018; Ramirez-Prado et al., 2018). The general trend emerging from many studies is that DNA methylation negatively regulates plant defense; in other words, loss of DNA methylation correlates with enhanced resistance (López Sánchez et al., 2016)]. This is illustrated by the fact that several Arabidopsis mutants depleted in DNA methylation, such as *met1-3* or the *drm1 drm2 cmt3* (*ddc*) triple mutant, showed stronger resistance to the bacterial pathogen *Pseudomonas syringae* (Pst) and increased expression of defense-related genes compared with Columbia-0 (Col-0) wild-type plants (Dowen et al., 2012; Espinas et al., 2016). This is in spite of these mutants having fundamentally different DNA methylation patterns: while *met1-3* has severely reduced global CG methylation, *ddc* is depleted of CHG and - to a



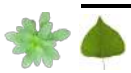
lesser extent - CHH methylation (Stroud et al., 2013), suggesting that there might be a hub at which these two DNA methylation pathways converge in the context of plant defense. In line with this idea, *met1 nrpd2* double mutants (NRPD2 is a subunit of RNA polymerase IV, a central component of the RdDM pathway), displayed enhanced resistance to *Pst* (Yu et al., 2013). The same study showed that treatment of Arabidopsis with the bacterial flagellin-derived peptide FLG22 caused downregulation of key components of the RdDM pathway (Yu et al., 2013), resulting in decreased CHH methylation levels. Together, these data indicate that DNA de-methylation - potentially in combination with the deposition of specific histone marks - might be a priming signal that predisposes defense-related genes to be rapidly expressed during an ensuing infection by the same or another pathogen. Following that rationale, methylation-deficient mutant plants would be "mimicking" this primed state and thus respond more rapidly and/or more efficiently when exposed to a pathogen. The same group recently showed that ROS1 is necessary to prevent gene-silencing RdDM from spreading from proximal TEs into the promoter of the defense-related gene *RECEPTOR-LIKE PROTEIN 43*; failure to do so resulted in masking of the binding site of PAMP-responsive WRKY transcription factors (Halter et al., 2021; Zervudacki et al., 2018).

But the relationship might be more complicated than a strict promotion of defense by hypo- or de-methylation. López Sanchez and colleagues observed antagonistic effects of *Hyaloperonospora arabidopsidis* (*Hpa*; downy mildew) infection in hyper- and hypo-methylated mutants of *A. thaliana*: on the one hand, hypo-methylated *nrpe* mutants (impaired in RNA Polymerase V and hence in RdDM) were more tolerant to *Hpa*, the hyper-methylated *ros1* mutant was more susceptible (López Sánchez et al., 2016). These antagonistic



responses correlated with opposite expression patterns of SA-dependent genes, up-regulated in *nrpe* vs. *ros1*. However, the effect was inverse for infections with the necrotrophic pathogen *Plectosphaerella cucumerina*, with *nrpe* being more susceptible and with JA-induced genes down-regulated compared to *ros1*. Whether JA-responsive genes are a direct target of DNA methylation or whether a simultaneous up-regulation of SA-responsive genes, which act antagonistically to the JA response, requires further investigation.

One might argue that it might be difficult to draw causal relationships by studying epigenetic pathway mutants with pleiotropic phenotypes. Epigenetic recombinant inbred lines (epiRILs) were established more than a decade ago in the model plant *A. thaliana* by crossing wild-type Col-0 to either *met1* (Reinders et al., 2009) or *ddm1* (Johannes et al., 2009) (the latter a loss-of-function mutant of the chromatin remodeler DECREASE IN DNA METHYLATION 1 with a similarly depleted global methylation as *met1*). Descendants of this cross are near-isogenic but show individual mosaic DNA methylation patterns (Catoni & Cortijo, 2018; Cortijo et al., 2014) epiRILs responded differently to JA and SA and varied in susceptibility to *Pst* infection, suggesting an underlying epigenetic cause (Latzel et al., 2012, 2013; Y.-Y. Zhang et al., 2018) Compelling evidence came from a recent study by Furci and colleagues: by measuring infection outcome with the biotrophic oomycete (*Hpa*) on more than 100 epiRILs from the Col-0 x *ddm1* cross, the authors identified four epigenetic quantitative trait loci (epiQTLs) explaining more than 60% of the variance in susceptibility (Furci et al., 2019; Figure 1A,B). Lines carrying a hypomethylated allele of these epiQTLs displayed a primed state of many defense-related genes, but few of these genes were contained in the actual mapping intervals, suggesting regulation *in trans* by still unresolved mechanisms (Figure 1C).

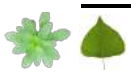


Associations between loss of DNA methylation and plant defense are not limited to Arabidopsis: hypomethylated rice plants treated with the DNA demethylating agent 5-azadeoxycytidine (5-aza) showed enhanced resistance to the bacterial pathogen *Xanthomonas* (Akimoto et al., 2007). Recently, it was shown that infection of diploid wheat *Aegilops tauschii* with the biotrophic pathogen *Blumeria graminis* f. sp. *tritici* (Bgt) reduces ARGONAUTE4a levels as well as 24-nt siRNAs and CHH methylation in stress response genes that are in close proximity to TEs (Geng et al., 2019)].

DNA methylation is not only implicated in the interaction with fungi or bacteria; several studies have characterized its role in plant-nematode interactions (for a full review on the topic, see (Hewezi, 2020). Identification of differentially methylated regions (DMRs) in soybean in response to infection by the soybean cyst nematode *Heterodera glycines* revealed mostly hypo-methylation in the plant root (Hewezi et al., 2017; Rambani et al., 2015). A recent follow up study showed that some of these methylation changes affected the expression of plant microRNA (miRNA) genes (Rambani et al., 2020), of which at least one played a role in the plant's response to the nematode.

DNA METHYLATION IN BENEFICIAL INTERACTIONS

Recent studies highlighted the role of DNA methylation in symbiotic and commensal interactions (Zogli & Libault, 2017). In the legume *Medicago truncatula*, the homolog of Arabidopsis *DEMETER*, *MtDME*, is expressed in the differentiation zone of forming nitrogen-fixing nodules that arise from the symbiosis between *M. truncatula* and a *Sinorhizobium* bacterium. Suppression of MtDME prevented the formation of nodules and led to transcriptional misregulation of several hundred nodulation-related genes,

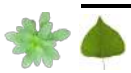


including many members of the nodule-specific cysteine-rich (*NCR*) gene family that are essential for nodule formation (Satgé et al., 2016). In line with this, the successive endopolyploidization that is a hallmark of nodule formation correlated with DNA methylation changes in about half of all *NCR* genes (Nagymihály et al., 2017); a directional correlation between hyper-/hypo-methylation and transcriptional down-/up-regulation was not detectable. Because this study used reduced-representation bisulfite sequencing (RRBS), the full extent or directionality of the methylation changes might have remained unclear. However, a recent study in soybean elucidated the association between DNA methylation, gene expression and alternative splicing in soybean nodules development and identified CHH hyper- and CHG hypo-methylation as prime features of the nodule methylome (Niyikiza et al., 2020).

Very little and partially contradicting information is available on the role of epigenetic mechanisms in mycorrhizal symbiosis. According to two reports, colonization of two *Geranium* species by arbuscular mycorrhizal fungi (AMF) caused increased DNA methylation levels in the host (Varga & Soulsbury, 2017, 2019). In contrast, AMF colonization in sunflower (*Helianthus annuus*) led to a specific transcriptional activation of certain *Copia* retroelements, suggesting a loss of suppressive DNA methylation at these loci (Vangelisti et al., 2019). All of these studies had limited power to resolve DNA methylation changes at the genomic level but might serve as a starting point for more detailed future analyses.

Beyond bilateral interactions, it is interesting to ask whether the epigenetic setup of the host influences or reacts to the more general state of plant-microbe associations. The advent of high-throughput sequencing has enabled the quantitative and qualitative assessment of the holobiont, the combination

of the host and all of its associated microorganisms (Vandenkoornhuyse et al., 2015). Even though to date there does not seem to be a systematic investigation of such associations, first studies in this area provide intriguing insights that "methylation moulds microbiomes" (Wilkinson & Ton, 2020). First evidence came from near-isogenic *A. thaliana* plants that had been regenerated via somatic embryogenesis and that carried epialleles related to the regeneration process and the tissue of origin of the embryos (Wibowo et al., 2018). Plants regenerated from root-derived somatic embryos showed significantly different association with bacteria, both with regard to natural communities in soil and after inoculation with synthetic communities (Wibowo et al., 2018). Vílchez and colleagues were able to establish a more direct association (Vílchez et al., 2020). Plants often shape the quantitative and qualitative composition of their microbiota by the release of specialized (also known as secondary metabolites)(Schandry & Becker, 2020). This study showed that the synthesis of myo-inositol production is under the antagonistic regulation of ROS1 and RdDM, and that active demethylation of biosynthesis genes is required for the establishment of beneficial *Bacillus megaterium* in the rhizosphere of *A. thaliana.*



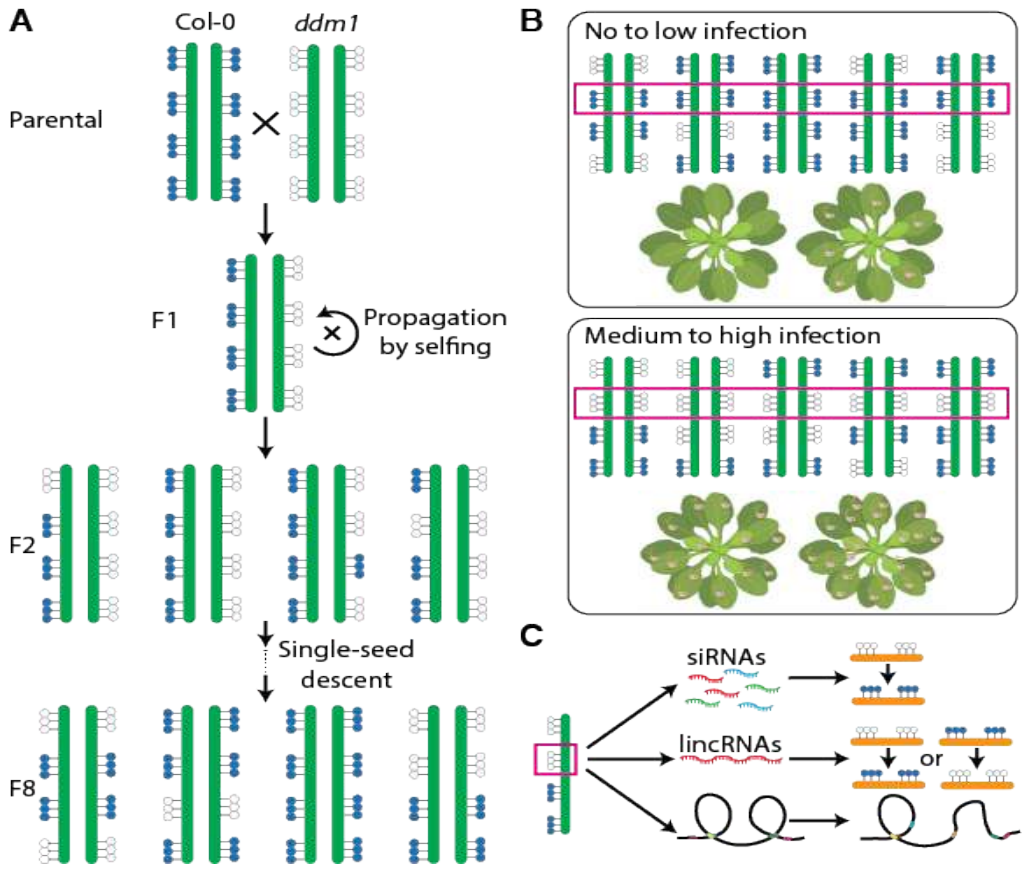


Figure 1. Locus-specific DNA hypomethylation promotes resistance to pathogens. **A)** Epigenetic recombinant lines (epiRILs) were derived from a cross of regularly methylated (blue hairpins) *A. thaliana* Col-0 wild-type with a methylation-depleted (white hairpins) *ddm1* mutant. For better visibility, methylation is indicated for only one chromatid of each homologous chromosome. F1 plants were selfed to generate several hundred F2 lines (Johannes et al 2009). Only lines carrying the homozygous *DDM1* wild-type allele were maintained. These lines were propagated for several generations by single-seed descent, resulting in lines with mosaic epigenotypes. **B)** In generation F9, resistance of 123 epiRILs to the oomycete *Hyalonospera arabidopsidis* (*Hpa*) was determined and leaves were divided into phenotypic groups based on the level of *Hpa* colonization. By statistically testing for associations between infection levels and the sequenced epigenotype of all lines, Furci and colleagues identified four

hypomethylated epigenetic quantitative trait loci (epiQTLs) correlating with elevated resistance; the red box schematically indicates one such epiQTL. **C)** Mapped epiQTLs regulate defense-related genes *in trans*. Here, three hypothetical ways of trans regulation are shown: short interfering RNAs (siRNAs) might arise from the hypomethylated epiallele and trans-regulate heterochromatin formation by sequence complementarity. By yet unresolved mechanisms, long intergenic non-coding RNAs (lincRNAs) can similarly change the chromatin configuration *in trans*. Ultimately, interactions between distant chromosomal loci could be altered by the change in the epigenetic configuration of one of these loci. Colored boxes are used to indicate physical loci.

SMALL RNAS IN CROSS-KINGDOM INTERACTIONS

In the context of the very complex defense system that plants have evolved to protect themselves, non-coding RNAs (ncRNAs) act as key players by reprogramming host gene expression in response to infection of a wide range of microbes and pathogens. Besides their role in antiviral defense, which is outside the scope of this review, ncRNAs of different types have been implicated in the response to pathogens.

Small RNA (sRNA) movement between hosts and interacting organisms can induce gene silencing through cross-kingdom trafficking. One of the most intriguing recent findings was that *A. thaliana* packages sRNAs into exosome-like vesicles. These vesicles accumulate at sites of infection by the fungal pathogen *Botrytis cinerea*. After fusion with the fungal cells, the vesicles deliver their content and the sRNAs inhibit infection-relevant genes to attenuate or even prevent the attack (Cai et al., 2018). However, pathogens have learned to make use of similar strategies by hijacking the plant's small RNA machinery. In a mechanism coined cross-kingdom RNA interference

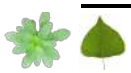
(ckRNAi), the oomycete *Hpa* releases sRNAs into the plant cell (*A. thaliana*) and utilizes plant ARGONAUTE1 (AGO1) to suppress plant immunity (Dunker et al., 2020). A complex sRNA-mediated interaction was described between *A. thaliana* and the hemibiotrophic oomycete pathogen *Phytophthora capsici* (Hou et al., 2019). Infection prompted locus-specific production of sRNAs that suppressed pathogen genes and thereby infection; however, the oomycete counteracts this plant defense by interfering with siRNA biogenesis. Altogether, these studies suggest that sRNAs, long thought to be exclusive antiviral agents, also act against microorganisms and that cross-kingdom RNAi is an essential element in plant immunity. Mobile mRNAs and miRNAs have also been implicated in plant-plant interactions; e.g., mobile mRNAs moving between both species were detected in Arabidopsis and tomato plants parasitised by the dodder species *Cuscuta pentagona* (Kim et al., 2014; LeBlanc et al., 2013). MiRNAs transferred from *C. campestri* to its host *A. thaliana* were shown to benefit the parasite (Alakonya et al., 2012). In the above examples, no altering of the chromatin state in either host or pathogen has been described, by which they do not fulfill the criteria of epigenetic regulation *sensu stricto*. It should be noted, however, that a systematic analysis of cross-kingdom epigenetic effects is lacking to date. Finally, while there are examples for sRNAs acting across *eukaryotic* kingdoms, their role in interactions between plants and *bacteria* remains unclear.

HISTONE MODIFICATIONS AS A REGULATORY ENTITY AND A TARGET

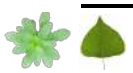
The third major epigenetic component are post-translational modifications (PTMs) of histones. Histone acetyltransferases (HATs) and deacetylases (HDACs), methyltransferases (HMT) and demethylases add or remove PTMs

at the N-terminal histone tails that protrude from the core histone octamer (Fan et al., 2015). Depending on the exact position of the amino acid that is modified and the type of modification, histone PTMs result in relaxed or condensed chromatin conformations, with the respective consequences for transcriptional activity. Histone acetylation favours an open chromatin configuration and therefore is associated with active transcription, while histone methylation can have an active or repressive effect on transcription depending on the residues that are modified (Garner et al., 2016). Because of the dynamic machinery of writers and erasers that set and remove such PTMs, histone modifications can change very rapidly, inducing global transcriptional changes (Table 1) or impacting transcription at a given genomic locus in response to an external signal such as an abiotic or biotic stress (Ashapkin et al., 2020; Berr et al., 2011)

In the context of biotic stress, both HATs and HDACs have been involved in plant defense response. For example, the elongator complex subunits ELONGATOR PROTEIN2 (ELP2) and ELP3, both of which have acetyltransferase activity, have been involved in the basal defense response and in ETI. Mutation in ELP3 caused higher susceptibility to *P. syringae pv. maculicola* (Psm) in Arabidopsis and a delay of defense gene induction following SA treatment or pathogen infection (DeFraia et al., 2013). Similarly, an ELP2 subunit is required for rapid defense gene induction upon infection with bacterial or fungal pathogens (DeFraia et al., 2010; C. Wang et al., 2015). Arabidopsis *elp2* mutants showed increased susceptibility to the necrotrophic fungi *B. cinerea* and failed to induce JA/ET defense pathway marker genes such as *PDF1.2*, *WRKY33* and *ORA59 (C. Wang et al., 2015)*. GCN5, another Arabidopsis HAT, regulated SA-mediated defense genes by acetylating H3K14 at their 5' and 3' ends (Kim et al., 2020). Several studies have explored



the role of HDACs in plant defense. Generally, HDACs have been described as negative regulators of SA-mediated defense response, since most of the plant HDAC mutants studied so far showed increased resistance to diverse pathogen infections (Ding & Wang, 2015; Espinas et al., 2016). For instance, Arabidopsis mutant's *hda19* and *hda6* are more resistant to *Pst* and show increased expression of SA-related defense genes *PR1* and *PR2* (Choi et al., 2012; Y. Wang et al., 2017). Similarly, mutants for the SRT2 deacetylase showed resistance to *Pst* infection and increased *PR1* expression (C. Wang et al., 2010). In rice, silencing of the histone deacetylase 701 (HDT701) also caused elevated transcription of defense-related genes and a resistant phenotype to both *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) pathogens (Ding et al., 2012). Arabidopsis HDA9 negatively regulates plant defense via the regulation of nucleotide-binding leucine-rich repeat (NLR) gene expression. Double mutants of HDA9 and its interaction partner HOS15 (*hda9 hos15)* showed enhanced resistance to *Pst* and increased expression of the NLR gene *SCN1* under infection conditions (Yang et al., 2020). In line with the role of HDACs as negative regulators of SA-mediated defense response, NO-mediated inhibition of HDACs increased acetylation in many genes involved in plant defense, including several SA defense genes (Mengel et al., 2017). Recently, another study uncovered a function of histone H2B in regulating bacterial defense when showing that H2B formed a regulatory module with the MAP kinase MPK3 that induced global genome acetylation changes during plant defense signaling (Latrasse et al., 2017). This study provided a mechanistic model for protein kinase signaling and its direct impact on chromatin landscape upon pathogen signaling. Contrary to the described resistance phenotypes of HDAC mutants, *h2b* mutants presented increased susceptibility to *Pst* (Latrasse et al., 2017). In a very different scenario, HDACs become the target of the organismic interaction:



benzoxazinoids, plant derived secondary metabolites that are produced in many grass species, can inhibit the growth of neighbouring species. The benzoxazinoid-derived compound 2-amino-3H-phenoxazin-3-one (APO) inhibited not only root growth of *A. thaliana* but also HDAC activity *in vitro* and caused global increase of histone H3 acetylation and altered gene expression (Venturelli et al., 2015).

Histone methylation plays an ambiguous role in plant biotic interactions. Due to the dynamic role of methylation in regulating transcription, both histone methyltransferases and demethylases have been proposed as positive or negative regulators of plant defense. The Arabidopsis demethylases JUMONJI 27 (AtJMJ27), AtJMJ14 and the INCREASE IN BONSAI METHYLATION 1 (IBM1), as well as the rice demethylase OsJMJ704 are positive regulators of defense. AtJM27 and OsJMJ704 suppress the transcription of master negative defense regulators: AtJMJ27 represses WRKY25 by removing H3K9me1/2; OsJMJ704 represses OsWRKY66, OsNRR, and Os11N3 via H3K4me2/3 demethylation (Chan & Zimmerli, 2019; Dutta et al., 2017; Hou et al., 2015; Li et al., 2020), while AtJMJ14 and IBM1 are required for the expression of several defense genes involved in local and systemic defense (Chan & Zimmerli, 2019; Li et al., 2020). The methyltransferases SDG8 and SDG25 have been implicated in PTI, ETI and systemic acquired resistance (SAR) against bacterial and fungal pathogens. *Sdg8* and *sdg25* single as well as *sdg8 sdg25* double mutants displayed increased susceptibility to both *B. cinerea* and *Pst* (De-La-Peña et al., 2012; Lee et al., 2016). This susceptible phenotype was attributed to an altered global histone methylation profile at the carotenoid *CCR2* and *CER3* loci, involved in carotenoid and cuticular wax biosynthesis, respectively (Lee et al., 2016). Interestingly, H2B ubiquitination was reduced at *CCR2*, *CER3*, and at

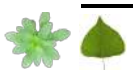
the H2B-monoubiquitinated resistance gene *SNC1* in *sdg* mutants, indicating crosstalk between the histone methylation and ubiquitination (Lee et al., 2016). Mutations in the methyltransferase gene *ATXR7* show reduced H3K4 methylation in *SNC1* and compromised resistance to *Hpa* (Xia et al., 2013). Another study highlighted the importance of the TE-mediated modulation of H3K9me2 levels at the *A. thaliana* disease resistance gene *RPP7*. The *COPIA-R7* retrotransposon inserted within this gene recruits the histone mark H3K9me2 to this locus, affecting the choice between two alternative *RPP7* polyadenylation sites in the pre-mRNA and thereby influencing the critical balance between two distinct transcript isoforms (Tsuchiya & Eulgem, 2013).

Several recent studies investigated the role of chromatin remodeling in plant defence. The Arabidopsis SWI/SNF class chromatin remodeling ATPase SPLAYED (SYD) is a positive regulator of defense against necrotrophs. Walley et al. showed that SYD regulates the JA/ET response during *B. cinerea* infection and is crucial for the defense response, while it was not required in defence against *Pst* (Walley et al., 2008). In *A. thaliana*, loss-of-function mutations in the PIE (PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1) and SWC6 (SWR1 COMPLEX 6) subunits of the chromatin remodeling complex SWR1c compromised basal resistance and ETI, while subunit ARP6 (ACTIN-RELATED PROTEIN6) loss of function enhanced it (Berriri et al., 2016). Another study showed the role of MICROCHORDIA (MORC) proteins in regulating chromatin accessibility during plant-pathogen interaction: *A. thaliana morc1/2* mutants infected with *Pst* showed an enriched proportion of differential DNase hypersensitive sites (DHS) at TEs, suggesting that MORCs modulate plant immune responses by binding to TEs, influencing both their expression and that of proximal genes following pathogen infection (Bordiya et al., 2016).

Table 1: Post-translational histone modifications in plant-organismic interactions.

Chemical modification	Histone modification	Observations	References
Acetylation	H3K9ac/H3K14ac	Histone acetyltransferase GCN5 regulates H3K14 acetylation levels at stress related genes	(Kim et al., 2020)
	H3K9ac	HDA9-HOS15 interaction regulates nucleotide-binding leucine rich repeat or Nod-Like Receptor (*NLR*) genes expression thorough H3K9ac deposition during defense response. Double mutants *hda9 hos15* showed enhanced resistance to *Pst.*	(Yang et al., 2020)
	H3K9ac and H3K14ac	Nitric oxide (NO) mediated HDAC inhibition regulates expression of defense related genes.	(Mengel et al., 2017)
	pan-H3ac	Allelopathic benzoxazinoid-derived compound 2-amino-3H-phenoxazin-3-one (APO)	(Venturelli et al., 2015)

		inhibits HDACs activity causing global increase of histone H3 acetylation and altered gene expression.	
	H3K9ac	Phytoplasma infection of Paulownia fortune alters transcriptional regulation.	(Yan et al., 2019)
	H4K12ac	Differential acetylation affects transcription of stress-responsive genes including resistant (R) proteins and stress related transcription factors in *Phaseolus vulgaris* infected with rust (*Uromyces appendiculatus*).	(Ayyappan et al., 2015)
	H3K9ac	Rice roots infected with nematode *Meloidogyne graminicola* show increased levels of H3K9ac at genes associated with defense response.	(Atighi et al., 2021)
Methylation	H3K4me1/2/3 and H3K36me1/2/3	Histone methyl transferases SDG8 and SDG25 regulate global defense gene expression affecting H3K4 and	(De-La-Peña et al., 2012; Lee et al., 2016)



	H3K36 methylation. Single and double mutants show increased susceptibility to both *Botrytis cinerea* and Pst.	
H3K4me2/3	Rice histone lysine demethylase Jumonji704 (JMJ704) controls defense response to bacterial blight disease. JMJ704 suppresses transcription of defense negative regulators genes such as NRR, OsWRKY62, and Os11N3 by reducing levels of the activating marks H3K4me2/3	(Hou et al., 2015)
H3K4me3 and H3K36me3	Altered transcriptional regulation of genes involved in metabolic pathways, biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, plant-pathogen interaction, and plant hormone signal transduction during	(Yan et al., 2019)

	phytpolasma infection in *Paulownia fortune*	
H3K4me2	Differential methylation affects transcription of stress-responsive genes including resistant (R) proteins, detoxifying enzymes, and genes involved in ion flux and cell death in *Phaseolus vulgaris* infected with rust (*Uromyces appendiculatus*).	(Ayyappan et al., 2015)
H3K27me3	Loss of the polycomb repressive complex protein LHP1 induces reduction in H3K27me3 levels at jasmonic acid (JA) and abscisic acid (ABA)-induced TFs ANAC019 and ANAC055 as well as some of their targets. Lhp1 mutants show increased aphid resistance, ABA sensitivity, and increased	(Ramirez-Prado et al., 2019)

	susceptibility to *Pseudomonas syringae* *(continued in next page)*	
H3K9me2/3	Dynamic histone regulation in root-knot nematode (*M. graminicola*) infection in rice. H3K27me3 is strongly enriched while H3K9me2 is generally depleted in galls formed in root upon infection. Differential histone peaks are associated with plant defense genes. Plants overexpressing two histone lysine methyltransferases (OsSDG729, OsSDG740) show contrasting susceptibility to *M. graminicola*.	(Atighi et al., 2021)

CONCLUSION

Besides host genotype (G), environment (E) and the interaction of both (G*x*E), host epigenotype is emerging as another component to be taken into consideration when investigating the relationship between plants and their biotic interactors. The examples provided here indicate that epigenetic regulation contributes to the outcome of plant defense, symbiosis, and parasitism. Future studies will be necessary to understand the molecular dependencies of the different layers of regulation and the impact of naturally occurring plant epigenetic variation on biotic interactions. Moreover, most studies to date have focused on the epigenetic configuration of the host, while there is limited data on epigenetic mechanisms or variation in the interactors, and how these might influence infection outcome. For example, in a rare investigation of epigenetic polymorphisms on the pathogen side, Wang et al showed that epiallelic H3K27me3 caused silencing of the *Phytophthora sojae* avirulence effector gene Avr1b and allowed evasion of the host immune recognition (Wang et al., 2020). Another major current limitation is that study systems have not been standardized in the field and often make it difficult to compare studies, either because different biotic interactors were chosen or because the general setup was different. Both of these hurdles can be overcome by future collaborative efforts between the fields of microbiology, plant immunity, and epigenetics.

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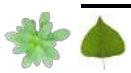
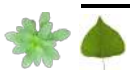


innovation programme via the Marie Sklodowska-Curie ETN 'EpiDiverse' (grant ID 764965).



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CHAPTER 2: VARIATION IN DNA METHYLATION AND RESPONSE TO SHORT-TERM HERBIVORY IN THLASPI ARVENSE

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ABSTRACT

Plant metabolic pathways and gene networks involved in the response to herbivory are well-established, but the impact of epigenetic factors as modulators of those responses is less understood. Here, we use the demethylating agent 5-azacytidine to uncover the role of DNA cytosine methylation on phenotypic responses after short-term herbivory in *Thlaspi arvense* plants that came from two European populations with contrasting flowering phenotypes expected to differ in the response to experimental demethylation. The experimental design followed a 2x3 factorial design that was replicated for each flowering-type. First, half the seeds were submerged in a water solution of 5-azacytidine and the other half only in water, as controls. Then, we assigned control and demethylated plants to three herbivory categories (i) insect herbivory, (ii) artificial herbivory, and (iii) undamaged plants. The effects of the demethylation and herbivory treatments were assessed by quantifying genome-wide global DNA cytosine methylation, concentration of leaf glucosinolates, final stem biomass, fruit and seed production, and seed size. For most of the plant traits analysed, individuals from the two flowering-types responded differently. In late-flowering plants, global DNA methylation did not differ between control and demethylated plants but it was significantly reduced by herbivory. Conversely, in early-flowering plants, demethylation at seed stage was still evident in leaf DNA of reproductive individuals whereas herbivory did not affect their global DNA methylation. In late-flowering plants, artificial herbivory imposed a stronger reduction than insect herbivory in global DNA methylation and final stem biomass, and induced higher concentration of aliphatic glucosinolates. In early-flowering plants, the effects of

herbivory were non-significant for the same traits. Finally, the effect of herbivory on reproductive parameters varied with the level of demethylation and the plant flowering-type. Although further investigations with more populations and families are required to confirm our results, they suggest that the genetic background of experimental plants and timing of damage can affect the response to herbivory and point towards multifaceted genetic-epigenetic interactions in determining herbivory-induced phenotypic plasticity.

INTRODUCTION

Interactions between plants and herbivorous insects are ubiquitous and these associations are diverse among biomes, communities and species (see e.g., (Fornoni et al., 2004; Moreira et al., 2018). Plants have evolved multiple traits to reduce herbivory, including mechanical and chemical defences and poor nutritional quality (Carmona et al., 2011). Some plant species also show the capacity to respond to herbivory through phenotypic plasticity. Such responses involve the activation of defence signalling pathways regulated by plant hormones such as jasmonic acid (JA), salicylic acid and ethylene (Pieterse & Dicke, 2007) and vary with timing, salivary factors associated to certain consumers, and amount of damage (see e.g.(Agrawal, 2000b; Bossdorf et al., 2004; Züst & Agrawal, 2017). In order for a response to become evolutionarily successful, it should reduce the impact on fitness even when incurring some costs (Cipollini et al., 2003; Douma et al., 2017). The magnitude of plastic phenotypic change varies across and within species and such variation could stem from both genetic and environmental factors (Josephs, 2018; Ogran et al., 2020; Wagner & Mitchell-Olds, 2018).

Besides genetic and environmental components, epigenetic factors such as DNA methylation, small RNAs and post-translational histone modifications have emerged as relevant modulators of plant responses to biotic challenges (Herrera et al., 2018; Ramos-Cruz et al., 2021). DNA methylation is the best studied epigenetic mechanism in plants and experimental evidence suggests its link to phenotypic plasticity. Experimental approaches to uncover the role of DNA methylation on ecologically relevant plant traits have often used DNA demethylating agents such as 5-azacytidine or zebularine (Alonso et al.,

2017; Fieldes et al., 2005; Latzel et al., 2020; Puy et al., 2018; Richards et al., 2010; Verhoeven & van Gurp, 2012). A combination of manipulation of DNA methylation with controlled herbivory experiments can be a fruitful strategy to elucidate the contribution of DNA methylation to specific plant responses after herbivory (Alonso et al., 2018, 2019). Although we focused on DNA methylation, it might be worth to indicate that 5-azacytidine is able to reduce cytosine methylation also in RNA. This aspect is out of our scope but it might contribute to plant response to stress (see e.g., (Tang et al., 2020).

Here, we studied the effects of 5-azacytidine and controlled herbivory on the performance and defence of the annual plant *Thlaspi arvense* L. (Brassicaceae) to evaluate the relationships between herbivory, phenotypic plasticity and epigenetic changes in plants. We selected this fast growing and widespread annual plant because many studies contributing to the molecular understanding of inducible defenses have been performed in the Brassicaceae (Rasmann et al., 2012; Lucas-Barbosa et al., 2017). This plant family is well characterized by the presence of specific specialized (or secondary) compounds, the glucosinolates (Halkier, 2016). These compounds are classified as aliphatic, indole and aromatic and, together with their breakdown products that are released upon tissue disruption, play an active role in plant defence (Wittstock et al., 2016; Züst et al., 2012). In particular, aliphatic glucosinolates are found in higher concentrations after herbivory or JA application, have a role in resistance against pest insects (Guo et al., 2013; Mikkelsen et al., 2003; Textor & Gershenzon, 2009) and their relative abundance can vary geographically within a species (e.g., Züst et al., 2012). Furthermore, *T. arvense* has agronomic value as

potential biofuel crop and it shows contrasting phenotypic traits associated to geographic origin and length of life-cycle that may be relevant to better understand variation in induced responses (Best & McIntyre, 1975; Moser et al., 2009; Royo-Esnal et al., 2015; Dorn et al., 2015; Claver et al., 2017).

Our experimental approach included demethylation with 5-azacytidine at seed stage (Alonso et al., 2017) and three different herbivory treatments: artificial leaf damage combined with JA-spraying, consumption by caterpillars of *Pieris brassicae* (Lepidoptera: Pieridae) and undamaged controls. The two herbivory treatments were expected to elicit analogous responses by dint of JA addition and the artificial one aimed to mimic consumption by any insect that could provide insight on generality of the observed responses (Züst & Agrawal, 2017). We estimated the genome-wide global DNA methylation level in leaves of reproductive adult plants and measured concentration of leaf glucosinolates, final stem biomass, fruit and seed production, and seed mass to test whether seed-stage demethylation influenced plant responses to short-term herbivory stress. As we were uncertain which plant-type would be more responsive to herbivory, we used seeds collected from two European populations that exhibited contrasting phenotypes, which roughly correspond to two formerly described strains and commercial varieties, namely early- and late-flowering types that differ in foliar and ecological traits (Royo-Esnal et al., 2015), and belonged to two different genetic clusters (Galanti et al., 2022). Our specific postulations were:

i. Experimental demethylation at seed germination will reduce leaf DNA methylation levels of reproductive adult plants of *T. arvense*, at least in late flowering phenotypes (Burn et al., 1993)

ii. Under controlled conditions, short-term herbivory will increase glucosinolates and can reduce plant fitness or not depending on tolerance (Núñez-Farfán et al., 2007; Textor & Gershenzon, 2009).

iii. Also, herbivory can alter DNA methylation, although the magnitude and sign of this latest effect is uncertain

iv. Altering DNA methylation will impair plant inducible defences (Latzel et al., 2020) and modify at least some of the plant responses after herbivory.

MATERIALS AND METHODS

STUDY SYSTEM

Thlaspi arvense L. (Brassicaceae) is a diploid annual species with a small genome size (1C = 539 Mb) (Johnston et al., 2005) and has a high degree of ecotypic variation, including variance in its life-cycle phenology and the need of previous vernalization to induce flowering (Burn et al., 1993). The wild population exhibit significant genetic diversity, phenotypic differentiation (Frels et al., 2019; Sedbrook et al., 2014) and epigenetic variation associated to environmental drivers (Galanti et al., 2022). Plants initially grow as a vegetative rosette, then bolt and produce racemes of flowers at the apices of the terminal and axillary branches. Plants of the early-flowering spring-type produce only a few leaves before internodes begin to elongate and individuals switch to reproductive growth, whereas the late-flowering winter-type forms a rosette of large leaves and requires vernalization to start flowering (Moser et al., 2009). The two flowering types have genetic and ecological differences (McIntyre & Best, 1978; Dorn et al., 2018). The species mainly self-pollinates and produces abundant siliques, each containing 10-20 seeds.

We used plants collected from two European populations with contrasting flowering phenotypes (Supplementary Material S1) and genetic backgrounds (Galanti et al., 2022). Plants collected near Uppsala (59°49'N, 17°39'E, 26 m a.s.l., in central Sweden) were late-flowering SE winter-type. Plants collected from Bossdorf (52°00' N 12°35'E, 151 m a.s.l., in north Germany) were early-flowering DE spring-type. The two populations occurred in roadsides and field margins, with a soil

depth of >50 cm. In the field, all the mature fruits from available individuals (N = 12-15) were collected from each population in July-August 2018 and stored in darkness for 3-4 months at room temperature. In the lab, we counted fruits and seeds of each sampled individuals and randomly selected three fruiting individuals per population, each having at least 30 mature fruits containing dark-brown seeds to obtain enough seeds for our experimental design (see below).

Insect herbivory assays were conducted by larvae of *Pieris brassicae* (L.), a specialist herbivore that feeds only on plants in the Brassicaceae (Lucas-Barbosa & Lucas-Barbosa, 2016). We obtained L2 instar larvae from a commercial supplier (www.lombricesdecalifornia.com), reared them on leaf cabbage and 1-2 days before the beginning of our experiments they were fed with leaves of *T. arvensis*.

EXPERIMENTAL DESIGN, GROWING CONDITIONS, AND TREATMENTS

We investigated the effects of experimental demethylation with 5-azacytidine at seed stage and leaf herbivory on plant performance, concentration of leaf glucosinolates, and epigenetic features of *T. arvensis* plants using an experimental design in which the two factors were crossed. More specifically, the leaf herbivory treatment had three levels: insect herbivory (hereafter named "INSH"), artificial herbivory ("ARTH") and undamaged controls ("CONH") and it was performed on young plants grown from seeds that were previously assigned to each of the two levels of the demethylation treatment: control (hereafter named "CON") and azacytidine-treated ("AZA").

Germination and plant growth were conducted in a growth chamber (Aralab CLIMAPLUS 400) at Doñana Biological Station, with long-day (LD) conditions: 16h of light at 22 ºC, and 8 h of darkness at 18 ºC, and 55% humidity. Chamber shelves were at a short distance (< 35 cm) to a combination of fluorescent cool-white and purple light tubes. Plants were regularly watered every 2 days during the germination period (the first 3 weeks), and every 3-5 days during the rest of the experiment. No fertilizer was applied during the whole experiment. In total, plants were grown during 14 weeks and first herbivory experiments were performed when plants were between 7 and 8 weeks old, closely before flowering for late-flowering SE and at the time of flowering onset for early-flowering DE.

Seed demethylation treatment. We selected 48 well-formed brown seeds per study plant (hereafter families). In total, 240 seeds were used to begin this experiment (2 population x 2-3 mother per population x 48 seeds per mother family). Seeds were surface-sterilized with a 5% bleach solution, washed with distilled water, individually scarified using clean sandpaper and placed in distilled water for 48 h at room temperature. For the demethylation treatment a 100 mM stock solution of 5-azacytidine (Sigma A2385–100mg) in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA) was prepared, stored at −20 °C, and diluted in water to 0.25 mM just before treatment (AZA). This nucleoside analogue incorporates into the genome of proliferating cells during DNA synthesis and traps DNA methyltransferases, targeting them for degradation and resulting in genome-wide demethylation (Lopez et al., 2016). In late-flowering SE *T. arvense*, this concentration has been reported as optimum to induce changes in methylation levels without

other nonspecific toxic effects (Burn et al., 1993). As a control, a mock solution of DMSO in water (3:97; v: v) was used (CON). Half of the seeds of each family were immersed respectively in AZA and CON solutions during 48 h at 4 ºC in darkness.

Seeds were then individually sown on commercial soil (Sustrato Universal El Clavel de Martínez SL) on 10 cm diameter pots. Groups of 14-15 pots were placed in trays and were randomized by the provenance of the seeds (families and populations). After three weeks, all emerged seedlings were subjected to vernalization at 5 °C during 21 d, with short day conditions (6 h of light and 16 h of darkness, watering once a week), to shorten the period of vegetative growth and synchronize flowering. We applied this protocol to all study plants because vernalization was known to reduce DNA methylation in *A. thaliana* (Burn et al., 1993). Thus, if applied only to late-flowering plants we could blur the consequences of vernalization and demethylation treatments applied to different sets of plants. For not having enough seeds per family, we could not include further replicated study factors. After vernalization, all the seedlings were re-transplanted into different pots using an enriched soil brand (Sustrato Universal Gramoflor Blumenerde) because of unsuitable quality of the previous one. Seedlings were allowed to grow during 2-3 weeks under LD conditions before further treatment.

Herbivory treatments. When plants were 7-8 weeks old, we selected up to 12 similar sized plants from each family and demethylation treatment, and randomly assigned four of them to each of the three herbivory treatments. Due to reduced germination rates such design was barely possible for two families in late-flowering SE and three families in early-

flowering DE. Altogether, we began this second part of the experiment with a total of 113 plants (46 from SE and 67 from DE, allocating 3-4 replicates per family in every group of treatment). In plants assigned to the ARTH treatment, simulated herbivory was induced by punching holes in two similar sized and well-developed leaves, and spraying a JA (Sigma J2500- 100MG) solution all over the leaves. JA was solubilized in ethanol, then diluted in distilled water to a 1 mM JA solution, and 0.1% triton-x 100 was added as a surfactant to increase penetration through the cuticle (Arnold & Schultz, 2002). In the INSH treatment, we selected two well-developed leaves of the plant and used small nylon mesh bags to individually encage two L4 instar larvae on each leaf. We let the larvae consume leaves for approximately 1-2 days, and then all the larvae were removed when 60-80% of each leaf was consumed. In the control treatment, two well-developed leaves were selected in each plant and sprayed with a control solution with the same composition as the JA solution except that it contained no JA. To ensure that all plants received a comparable amount of solution, each treated leaf was first sprayed with two pumps of a mechanical sprayer, and then the third pump was sprayed over the whole plant. After 10 days, a second bout of the same herbivory treatment was conducted on each plant to enable a priming effect and finally get a stronger and/or faster response (Rasmann et al., 2012; Mauch-Mani et al., 2017). Treated leaves of this second herbivory treatment were selected as close as possible to the first ones and from the same developed stage. During all the treatment phases, all plants including controls were covered with individual nylon mesh bags that were removed 48 h after the end of the treatment. Also, to avoid any interference of volatile compounds between plants with different treatments, plants subjected to either INSH or ARTH

treatments were stored in two separate chambers with identical conditions as controls (germinator MiniDiGiTII, Rabider) in LD conditions for 48 h.

Sample collection. Unwounded leaves from all individuals were collected and immediately frozen in liquid nitrogen 24 h after second artificial herbivory or larvae removal. Vials were kept at -80 °C until further processing. The experiment was finished in total 14 weeks after seed sowing, when fruits were mature, and individuals started to become senescent. All the aboveground biomass of each plant, including senescent leaves, fruits and stems, was collected in individually labelled paper bags and placed in well-aerated room until measuring stem biomass, and fruit and seed numbers.

DATA COLLECTION AND SAMPLE PROCESSING

Global DNA cytosine methylation

For each individual an aliquot of 30 mg leaf frozen material was homogenized to a fine powder using a Retsch MM 200 mill. Total genomic DNA was extracted using Bioline ISOLATE II Plant DNA Kit, which contain RNAse A to remove RNA from the samples, and quantified using a Qubit fluorometer 2.0 (Thermo Fisher Scientific, Waltham, MA, USA). 100-ng aliquot of DNA extract was digested with 3 U of DNA Degradase PlusTM (Zymo Research, Irvine, CA, USA), a nuclease mix that degrades DNA to its individual nucleoside components. Digestion was carried out in a 40 µl volume at 37 °C for 3 h, and terminated by heat inactivation at 70 °C for 20 min. Three independent replicates of digested DNA per sample were initially

processed to estimate global cytosine methylation more precisely; the number of replicates was increased for some individual samples to reduce inconsistencies; altogether 376 vials were processed in randomized order. Selective derivatization of cytosine moieties with 2-bromoacetophenone under anhydrous conditions and subsequent reverse phase high performance liquid chromatograph (HPLC) with spectrofluorimetric detection were conducted. The percentage of total cytosine methylation on each replicate was estimated as 100*5mdC/ (5mdC + dC), where 5mdC and dC are the integrated areas under the peaks for 5-methyl-2'-deoxycytidine and 2'-deoxycytidine, respectively (see Alonso et al., 2016 for further details).

Germination, seedling emergence and early developmental traits

Germination of field collected seeds from the two populations was monitored after planting for every two days during three weeks. After that time, germination was extremely rare. For each individual seed we recorded the dates of germination (radicle visible, i.e., at least 1mm long), seedling emergence (both cotyledons fully opened) and appearance of the first two leaves (completely expanded). Time to seedling emergence and appearance of the first leaf for each seedling was calculated from date of sowing. Germination probability was calculated as the total number of seeds germinated from the total number of seeds planted after three weeks of sowing. Flowering phenology was monitored with two censuses per week during 8-9 weeks, after that all plants were in bloom. Plant height (in cm) at the onset of flowering was measured as the length from cotyledon insertion to the apex of the main inflorescence.

Individual size and fecundity

. After harvesting, each plant was carefully separated into three different components: stems, leaves and reproductive organs (including fruits, seeds, perianth segments and bracts). Dry weight of each component was separately determined after oven-drying plants to a constant mass during at least 48 h at 40 °C using a digital balance to the nearest 0.01 mg. The stem biomass constituted the biggest portion of total biomass and was selected as the most informative trait because some old dry leaves and dry fruits were eventually detached from plants at harvesting.

Fruit production was estimated by taking each raceme individually and all mesocarps and bare pedicels attached to the plant were counted as fruits. Additionally, ten fully developed and mature fruits from each plant were randomly selected, including always fruits produced both in the main flowering stem and in its lateral branches. For each fruit we counted the number of sound seeds (fully developed and well-shaped) and unripe seeds (shrunken and markedly smaller in size) and then mean seed number per fruit was calculated. The total mass of all the sound seeds produced per fruit was weighed collectively in a digital balance to the nearest 0.01 mg and the average seed mass for each fruit was then estimated as group mass/no. seeds.

Glucosinolates

For glucosinolate (GLS) analyses, leaf material of two individuals per group of treatment and mother (N = 49) was shock-frozen in liquid nitrogen and lyophilized. The material was weighed and used for extraction of GLS, following the protocol by Abdalsamee and Müller (2012) (Abdalsamee & Müller, 2012). The dried material was

extracted threefold with 80 % methanol, adding *p*-hydroxybenzyl GLS (glucosinalbin, Phytoplan, Heidelberg, Germany) as internal standard at the first extraction. After centrifugation, supernatants were applied onto ion-exchange columns containing diethylaminoethyl (DEAE) Sephadex A25 (Sigma Aldrich, St.Louis, MO, USA) in 0.5 M acetic acid buffer, pH 5. Purified sulfatase was added to convert GSs to desulfoGLSs overnight. DesulfoGLSs were eluted in water and analyzed on a HPLC coupled to a DAD detector (HPLC-1200 Series, Agilent Technologies,Inc., Santa Clara, CA, USA). A gradient of water to methanol was used to elute desulfoGSs from a Supelcosil LC 18 column (3 µm, 150×3 mm, Supelco, Bellefonte, PA, USA). The gradient started at 5 % methanol, which was kept for 6 min and then increased from5 % to 95 % within 13 min with a hold at 95 % for 2 min, followed by column cleaning. GLSs were identified based on their retention times and UV spectra in comparison to respective standards (Fahey et al., 2001). Peak areas were integrated at 229 nm and the concentration of each of the four identified GLS compounds was calculated in relation to sample dry mass, using the following glucosinolates response factors: 1 for 2-propenyl-glucosinolate (sinigrin), 0.95 for benzyl-glucosinolate (glucotropaeolin), and 0.26 for both indol-3-ylmethyl-glucosinolate (glucobnsicin) and 4-methoxy-indole-3-yl glucosinolate (4-methoxyglucobrassicin). Total GLS concentration was estimated by summing all the concentrations for the four measured GLS compounds.

DATA ANALYSES

All statistical analyses were carried out using the R environment (R Development Core Team, 2020).Data distributions were visually inspected and absence of obvious outliers was confirmed. As a rule, we used linear or generalized linear mixed models to assess the sign, magnitude and statistical significance of the effects of demethylation and herbivory (fixed factors), accounting for the appropriate grouping random effects as defined in lmer and glmer functions of the lme4 library (Bates et al., 2015). Although a three-way factorial analysis that included population as a fixed factor would have been statistically more robust, we split the dataset by population to improve resolution because two reasons: firstly, graphical exploration of data distributions and model outputs suggested contrasting patterns that were not detected as significant interactions due to large variances and reduced sample sizes within groups, constraining greatly our statistical power; and secondly, some of the variables were far from a continuous normal distribution, actually they were nearly bimodal or had important discontinuities between populations, and therefore splitting the data improve models adjustment. Below we describe the models applied to each response variable type, which passed model diagnostic tests and were selected according to lower AIC (Bolker, 2015).

The model for the replicated global DNA cytosine methylation data was applied using *lmer*, it included demethylation, herbivory (with three levels) and their interaction as fixed effects, and plant as a random effect to correctly identify all replicates of the same sample. For germination analyses, every germinated seed was coded as 1 and every non-germinated seed coded as 0. Germination probability was modelled in *glmer* as a binomial process using logit as the link function, the model

included demethylation as the only fixed factor, and families (seeds coming from the same mother plant) as a random effect, which ensured that any possible influence of family heterogeneity in genetic background were adequately accounted for (i.e., blocked; Mead, 1988). Count variables such as days to seedling emergence and days to appearance of the first leaf were modelled as Poisson processes in *glmer* with logit link function. As these variables were measured before herbivory, models included only demethylation as fixed factor and families as random effect. Total fruit number was modelled also as a Poisson process with demethylation and herbivory as fixed factors and families as random effect using logit link function. Stem biomass, average seed number and average seed mass were modelled as Gaussian response variables in *lmer* including the same fixed and random factors.

We visualized glucosinolates profiles as nonmetric multidimensional scaling (NMDS) plots using Bray-Curtis dissimilarity index matrices. Difference in total glucosinolate profiles was analysed for herbivory and demethylation treatment using ANOSIM (function adonis2) with 9999 permutations in *vegan* (Oksanen et al., 2020). Further, variance in concentration of total glucosinolates, sinigrin and 4-methoxyglucobrassicin was analysed with *lmer*, models included demethylation and herbivory as fixed factors, and families as a random effect.

For each analysis, significance of fixed factors and their interaction was tested using the function Anova (package car; Fox & Weisberg, 2018), with type II sum of squares and the Kenward-Roger approximation to calculate the residual degrees of freedom. Estimated marginal means and associated confidence intervals for the response

variable at each factor level were obtained with the 'emmeans' function of the 'emmeans' library (Lenth R., 2018). Post hoc analyses were done by conducting multiple pairwise comparisons of the estimated marginal means with Tukey adjustment. Marginal means from generalized linear models were back transformed to the original scale of measurement. Mean ± SE will be shown unless otherwise stated.

RESULTS

GLOBAL DNA CYTOSINE METHYLATION

The percentage of cytosine methylation in leaf DNA of untreated *T. arvense* adult plants averaged 15.1 % (± 0.20, standard error). DNA methylation ranged between 13.8 and 16.5 % in control individuals (N = 15) and between 13.0 and 16.6 % in the full dataset (N = 91). The results of the full factorial ANOVA for global DNA methylation, stem biomass, reproductive traits and chemical defences are provided in the supplementary material Table S1.

The effect of demethylation and herbivory treatments on leaf DNA methylation at adult stage varied among the two study populations (Fig 1). In late-flowering SE, leaf DNA methylation did not differ between CON and AZA treated plants. In contrast, leaf DNA methylation was significantly affected by herbivory (χ^2 =6.52, df = 2, P = 0.038), methylation being higher in CONH plants (15.3 % ± 0.2) and more significantly reduced in plants assigned to ARTH (14.5 % ± 0.2) than those consumed by insect herbivores (14.8 % ± 0.2). In early-flowering DE, leaf DNA of AZA treated plants had slightly lower methylation levels than the CON plants (14.4 % ± 0.2 *vs.* 14.9 % ± 0.2, respectively;

χ^2 = 3.48, df = 1, P = 0.06), whereas herbivory did not affect DNA methylation levels of collected leaves.

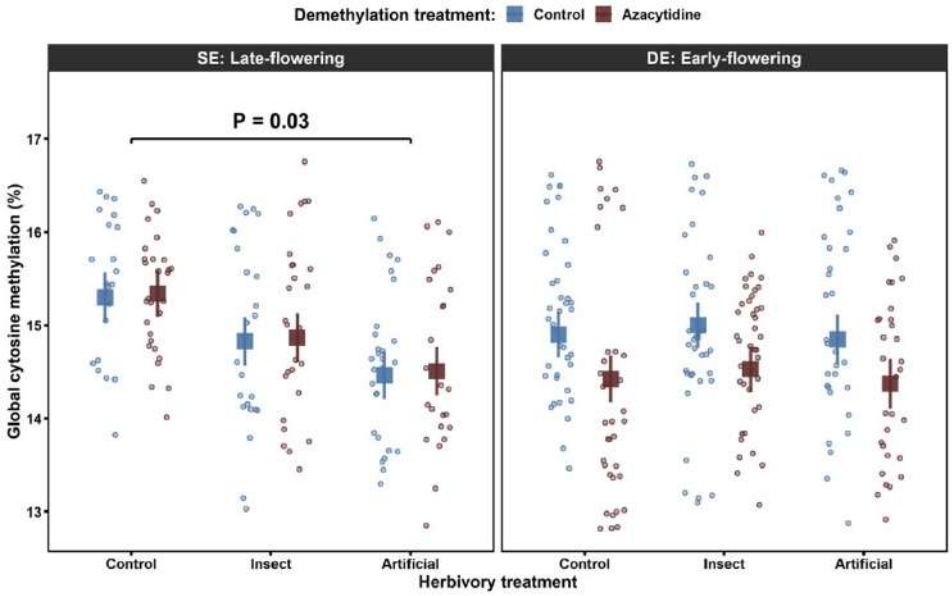


Figure 1. Variation in global cytosine methylation levels for plants (N = 374 data points, 3-6 replicates in 91 plants) of the two study populations (late-flowering SE and early-flowering DE), the three levels of our herbivory treatment (control, insect and artificial herbivory) and the two levels of our demethylation treatment (controls –in dark blue-, and azacytidine –in dark red-). Solid squares with bars show estimated marginal means and standard errors (from linear mixed-effects models, with the interaction "herbivory×demethylation" effect included, and "plant" as a random effect) across each level of the two treatments in the two study populations. The only contrast with significant P-value obtained for the comparison between artificial herbivory and control plants in late-flowering SE population, is shown. In early-flowering DE population, the contrasts obtained for every azacytidine *vs* control comparison were marginally significant (P = 0.06).

Germination probability was similar in the two study populations (0.732 ± 0.06 and 0.764 ± 0.05, for SE and DE respectively). Demethylation treatment did not affect it (SE: $\chi^2 = 0.21$, df = 1, P = 0.646; DE: $\chi^2 = 1.01$, df = 1, P = 0.314).

On average, seedlings treated with AZA emerged one day later and needed 2.5 days more to produce their first leaf than CON seedlings (Table 1). But, the effect of the demethylation treatment was slightly different in the two populations. The effect of AZA was not statistically significant for seedling emergence of the late-flowering SE plants ($\chi^2 = 0.75$, df = 1, P = 0.39) and showed only a near significant effect on first leaf development time ($\chi^2 = 3.15$, df = 1, P = 0.060). Whereas, in early-flowering DE plants the delay was statistically significant for both seedling emergence ($\chi^2 = 5.36$, df = 1, P = 0.020) and the appearance of the first leaf ($\chi^2 = 9.76$, df = 1, P = 0.001).

At the onset of flowering, just before herbivory treatment, again only in early-flowering DE plants the demethylation treatment produced a significant effect, being AZA treated plants almost 2 cm shorter than control ones ($\chi^2 = 4.45$, df = 1, P = 0.04). The same treatment had no effect on plant height of late-flowering SE plants (Table 1).

Table 1. Time (in days) from seed sown to seedling emergence and to the appearance of the first two leaves (first leaf emergence), and height (in cm) of plants at the onset of flowering (height at flowering) from control and 5-azacytidine treated seeds of the two *T. arvense* populations studied (late-flowering SE and early-flowering DE). Values shown are model estimated marginal means and their standard errors (in brackets). Only significant differences ($P < 0.05$) between controls and azacytidine treated plants are shown (P values).

Trait	Population	Control	Azacytidine	P value
Seedling emergence (d)	SE	5.85 (0.72)	6.50 (0.76)	
	DE	5.00 (0.51)	6.35 (0.60)	0.02
First leaf emergence (d)	SE	12.25 (0.78)	14.34 (0.79)	
	DE	14.96 (0.81)	18.20 (0.92)	0.002
Height at flowering (cm)	SE	13.00 (0.67)	12.80 (0.67)	
	DE	14.60 (0.83)	13.00 (0.82)	0.04

FINAL STEM BIOMASS

Final stem biomass in untreated (non-demethylated and undamaged) plants of late-flowering SE was on average two-fold higher than those of early-flowering DE (1.06 g ± 0.04 and 0.46 g ± 0.05, for SE and DE respectively), with almost no overlapping in figures obtained for each population (Fig. 2), and this difference was statistically significant (t-test: $t = 8.03$, $df = 18$, $P < 0.001$).

In late-flowering SE, variance in the final stem biomass was explained by a significant effect of herbivory ($\chi^2 = 10.25$, $df = 2$, $P = 0.005$), there was no effect of demethylation and the interaction between main factors was also not significant (Table S1). Specifically, ARTH treated plants had a strong and significant reduction in the stem biomass when compared to CONH and INSH plants, regardless of their initial seed demethylation treatment (Fig. 2). Further, stem biomass was higher in AZA treated plants without herbivory but lower in those which were subjected to insect herbivory.

In early-flowering DE, there was no significant effect of demethylation or herbivory treatment or their interaction on the final stem biomass (Fig. 2; Table S1).

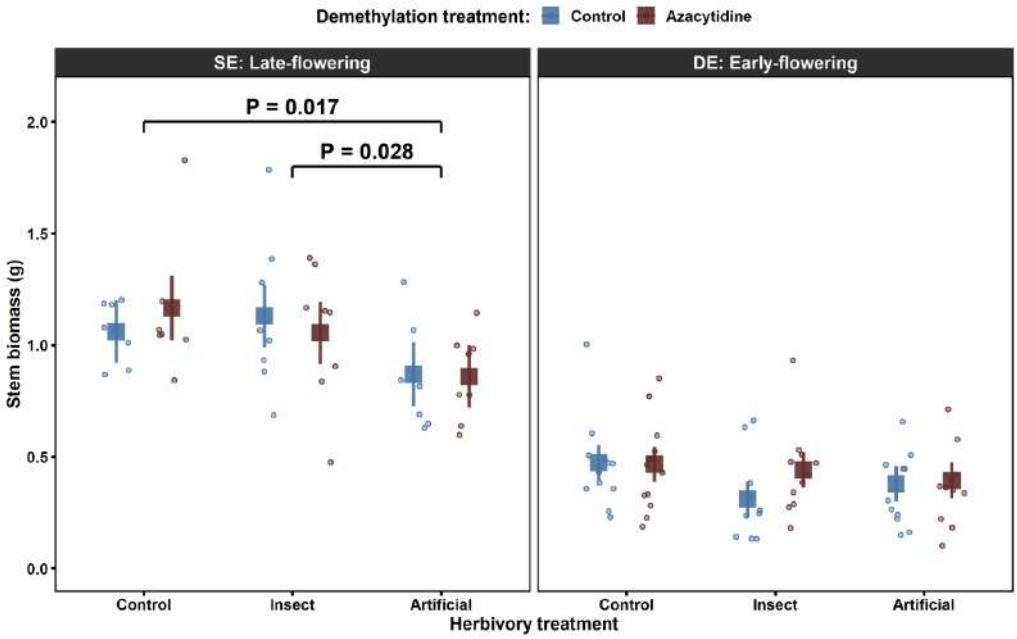


Figure 2. Variation in stem biomass for plants (N = 113 individual plants) of the two study populations (late-flowering SE and early-flowering DE), the three levels of our herbivory treatment (control, insect and artificial herbivory) and the two levels of our demethylation treatment (controls –in dark blue-, and azacytidine –in dark red-). Solid squares with bars show estimated marginal means and standard errors (from gaussian general linear mixed-effects models, with the interaction "herbivory×demethylation" effect included, and "plant" as a random effect) across each level of the two treatments in the two study populations. Contrasts with significant P-values ($P < 0.05$), obtained only for control *vs* artificial and insect *vs* artificial comparisons in late-flowering SE population, are also shown.

TOTAL FRUIT NUMBER

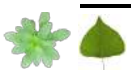
Thlaspi arvense plants grown in pots produced between 57 and 176 fruits for untreated plants (N = 20) and ranged between 23 to 198 in the full data set (N =112). Total fruit number in untreated plants of late-flowering SE was on average higher by ten fruits than those of early-flowering DE (108.75 ± 35.59 and 98.66 ± 29.15, for SE and DE respectively).

In late-flowering SE, variance in total fruit number was explained by a significant effect of herbivory treatments ($\chi^2 = 13.36$, df = 2, $P < 0.001$). Demethylation and the interaction between the two factors were not significant (Table S1). In particular, plants that experienced INSH produced a significantly lower number of fruits than plants that experienced no herbivory and ARTH, although these differences seem to be significant only for plants without AZA treatment (CON) (Table 2).

In early-flowering DE, total fruit production differed for demethylation ($\chi^2 = 9.56$, df = 1, $P < 0.001$) and herbivory treatments ($\chi^2 = 5.89$, df = 2, P = 0.052). The AZA treated plants produced more fruits, the impact being stronger in both ARTH and INSH plants (Table 2).

AVERAGE SEED NUMBER PER FRUIT

The average number of seeds per fruit ranged between 4.5 and 11.6 for untreated individuals (N = 20) and it was between 3.1 and 11.7 in the full data set (N = 112). a Untreated late-flowering SE plants produced more seeds per fruit than early-flowering DE plants (9.81 ± 1.87 and



6.89 ± 1.05, for SE and DE respectively; t-test: t = 3.99, df = 9.96, P = 0.002).

In late-flowering SE, the demethylation treatment had a significant effect on average seed number per fruit (χ^2 =7.45, df = 1, P = 0.006). Herbivory and the interaction between the two factors were not significant (Table S1). AZA treated plants produced significantly lesser number of seeds per fruit and this effect was particularly large in the group of undamaged controls (Table 2).

In early-flowering DE, average seed number per fruit varied significantly with demethylation (χ^2 = 12.45, df = 1, P<0.001), herbivory treatment (χ^2 = 7.88, df = 2, P = 0.02) and there was no significant interaction between the two experimental factors (Table S1). In this case, AZA treated plants produced higher number of seeds per fruit than their controls in all the three herbivory treatments, but the effect was particularly large in artificially damaged individuals (Table 2).

AVERAGE SEED MASS

In *T. arvense,* average seed mass ranged between 0.65 mg to 1.11 mg in untreated individuals (N = 20) and it ranged between 0.09 mg to 1.32 mg for entire data set after removing individuals with outlier values (N = 113). Average seed mass in untreated plants was similar in the two study populations (0.82 ± 0.08 mg and 0.90 ± 0.15 mg, for SE and DE respectively).

In late-flowering SE, a significant demethylation treatment effect was observed (χ^2 = 3.88, df = 1, P = 0.048). But herbivory treatment or interaction between two factors had no significant effect on average

seed mass (Table S1). Initial demethylation reduced the average seed mass per fruit in all the levels of our herbivory treatment, this effect was particularly large in insect consumed plants (Table 2).

In early-flowering DE, a significant and stronger effect of demethylation (χ^2 = 10.81, df = 1, P < 0.001) and a near significant interaction between the two experimental factors (χ^2 = 5.61, df = 2, P = 0.06) were found.

Table 2. Total fruit number, average seed number per fruit and average seed mass (mg) produced by control and 5-azacytidine treated plants after each of the three levels of herbivory treatment (undamaged control, insect herbivory, artificial herbivory) of the two *T. arvense* populations (late-flowering SE and early-flowering DE). Values shown are model estimated marginal means and their standard errors (in brackets). Only significant differences ($P < 0.05$) for each of the contrasts between controls and 5-azacytidine treated plants are shown (P values). Values with different letters indicate significant differences ($P < 0.05$) between the three levels of the herbivory treatment for each population and each level of demethylation treatment.

Trait	Population	Herbivory	Control	Azacytidine	P value
Total fruit number	SE	Control	106.75 (15.03)[a]	103.35 (14.62)	
		Insect	88.59 (12.55)[b]	95.83 (13.54)	
		Artificial	100.62 (14.25)[a,b]	94.97 (13.42)	
	DE	Control	99.85 (7.76)	104.30 (8.15)	
		Insect	90.37 (7.12)	99.58 (7.87)	0.032
		Artificial	95.06 (7.49)	104.67 (8.18)	0.027
Average seed number	SE	Control	9.81 (0.78)	7.01 (0.84)	0.018

		Insect	8.74 (0.78)	7.00 (0.78)	
		Artificial	9.00 (0.84)	8.21 (0.78)	
	DE	Control	6.89 (0.39)	7.58 (0.39)	
		Insect	5.69 (0.45)	6.63 (0.41)	
		Artificial	6.04 (0.41)	7.84 (0.41)	0.002
Average seed mass (mg)	SE	Control	0.82 (0.07)	0.70 (0.08)	
		Insect	0.92 (0.07)	0.74 (0.07)	
		Artificial	0.76 (0.08)	0.70 (0.07)	
	DE	Control	0.90 (0.08)[a]	0.99 (0.08)	
		Insect	0.71 (0.08)[b]	1.02 (0.08)	0.0003
		Artificial	0.87 (0.08)[a]	0.94 (0.08)	

In contrast to SE, the AZA treated plants produced heavier seeds than their controls, and again this effect was stronger for insect consumed plants (Table 2).

LEAF GLUCOSINOLATES

Total glucosinolate concentration in unwounded leaves collected 24 h after the second herbivory event varied between 1.2 μmol.g−1 d.w. to 1.7 μmol.g−1 d.w. in untreated individuals (N = 8) and it ranged between 1.2 μmol.g−1 d.w. to 39.4 μmol.g−1 d.w. for the entire data set (N = 49). The glucosinolate profiles were studied by NMDS using the concentrations of the two indole and two aliphatic compounds that were most abundant. The ANOSIM test showed that herbivory treatments exhibited a significant effect for late-flowering SE plants ($P < 0.005$) and not significant for early-floweirng DE plants ($P = 0.07$). The effect of demethylation was not statistically significant to explain variance in multidimensional glucosinolate profile. In late-flowering SE, variance in total GLS concentration was significantly explained by demethylation ($\chi^2 = 5.14$, df = 1, $P = 0.02$), herbivory ($\chi^2 = 40.37$, df = 2, $P < 0.001$) and interaction ($\chi^2 = 7.73$, df = 2, $P = 0.02$). The total GLS concentration was higher in leaves of ARTH plants, intermediate in INSH and significantly lower in undamaged CONH plants, specifically for plants which had undergone seed-stage AZA treatment (Table 3). In early-flowering DE, no significant effect of herbivory or demethylation was observed.

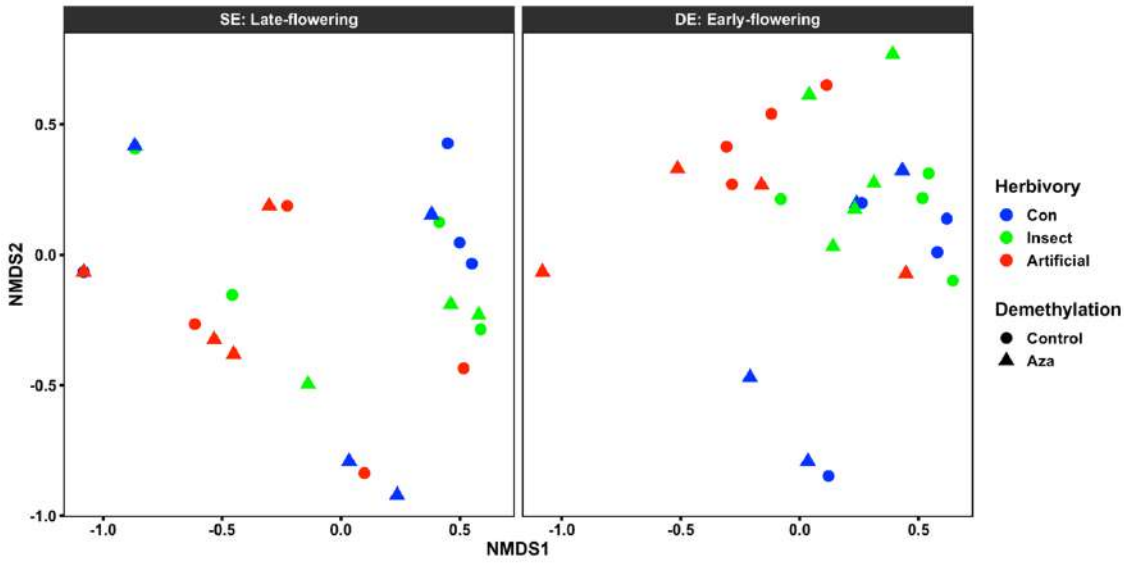


Figure 3. Nonmetric multidimensional scaling (NMDS) plots for glucosinolate profiles in *Thlaspi arvense* leaves in the two study populations (late-flowering SE and early flowering DE, N=49 individual plants). Colors denote herbivory treatments (controls -in blue-, insect herbivory -in green-, and artificial herbivory -in red-) and shapes denote demethylation (CON -triangle- and AZA -round-). The composition and concentration of the main four glucosinolate were analysed to distinguish their clustering patterns among herbivory and demethylation treatments.

The aliphatic GLS sinigrin was predominant and accounted for more than 98% of total GLS amount. The effect of experimental treatments on concentration of sinigrin, and the most abundant indole glucosinolate, 4-methoxyglucobrassicin, were also analyzed. In late-flowering SE, variance in leaf sinigrin concentration among individual plants was significantly explained by herbivory (χ^2 =38.95, df = 2, P < 0.001), demethylation (χ^2 = 5.06, df = 1, P = 0.02), and the interaction between the two experimental factors (χ^2 = 7.77, df = 2, P = 0.02). In

regards of herbivory, we found that the sinigrin concentration in leaves of ARTH plants were on average 3-10 fold higher than in leaves of plants assigned to INSH and CONH (Table 3). The AZA treatment led to a higher concentration of sinigrin, although the difference was only significant in leaves of ARTH plants (Table 3). In early-flowering DE, no significant effect of herbivory or demethylation was observed.

Table 3. Concentrations (expressed in μmol/g of dry weight) of total glucosinolates (Total GLS), 2-propenyl-glucosinolate (sinigrin), and 4-methoxy-indole-3-yl glucosinolate (4-methoxyglucobrassicin) detected in samples collected from leaves of control and 5-azacytidine treated plants of the two *T. arvense* populations (late-flowering SE and early-flowering DE) 24 h after the end of our herbivory trial. Values shown are model estimated marginal means and their standard errors (in brackets). Only significant differences ($P < 0.05$) for each of the contrasts between controls and 5-azacytidine treated plants are shown (P values). Values with different letters indicate significant differences ($P < 0.05$) between the three levels of the herbivory treatment for each population and each level of demethylation treatment.

Trait	Population	Herbivory	Control	Azacytidine	P value
Total GLS	SE	Control	1.68 (2.08)[a,b]	1.55 (2.08)[a]	
		Insect	0.97 (2.26)[a]	3.20 (2.08)[a]	
		Artificial	7.35 (2.26)[b]	16.28 (2.52)[b]	0.004
	DE	Control	1.24 (4.32)	2.04 (4.32)	
		Insect	5.82 (4.32)	4.80 (4.07)	
		Artificial	7.82 (4.75)	10.87 (4.75)	

Sinigrin	SE	Control	1.64 (2.14)	1.50 (2.14)[a]	
		Insect	0.88 (2.31)	3.07 (2.14)[a]	
		Artificial	7.18 (2.31)	16.25 (2.58)[b]	0.004
	DE	Control	1.20 (4.32)	2.03 (4.32)	
		Insect	5.77 (4.32)	4.78 (4.07)	
		Artificial	7.81 (4.75)	10.82 (4.75)	
4-methoxyglucobrassicin	SE	Control	0.03 (0.04)	0.04 (0.04)	
		Insect	0.06 (0.04)	0.13 (0.04)	
		Artificial	0.09 (0.04)	0.04 (0.04)	
	DE	Control	0.03 (0.01)	0.01 (0.01)	
		Insect	0.03 (0.01)	0.02 (0.01)	
		Artificial	0.01 (0.03)	0.04 (0.02)	

Variation in concentration of the most abundant indole GLS, 4-methoxyglucobrassicin was only explained by the experimental treatments in SE population. The effects of herbivory treatment (χ^2 = 6.20, df = 2, P < 0.04) and interaction of demethylation and herbivory (χ^2 = 6.18, df = 2, P = 0.04) were statistically significant in late-flowering SE (Table 3). Leaves of AZA treated plants had a significantly lower concentration of 4-methoxyglucobrassicin after artificial herbivory but a higher concentration in those plants consumed by insects (Table 3). In early-flowering DE, similar to the response of other GLS, the effects of experimental treatments did not significantly explained variance in concentration of 4-methoxyglucobrassicin (Table 3).

DISCUSSION

In this study, we combined experimental herbivory with experimental demethylation in *T. arvense* aiming to elucidate the role of epigenetic variation on short-term defence and plant performance of this annual herb under controlled conditions. This approach has been successfully applied to other study systems although mainly to analyze the responses to abiotic stress (see e.g., (Herman & Sultan, 2016; Latzel et al., 2012). To better interpret the obtained results it is important to emphasize that the two study populations exhibited contrasting phenotypes (Supplementary material, S1) and belonged to different genetic clusters within Europe (Galanti et al., 2022). Phenotypic differences were reduced because all individuals were vernalized as seedlings and, thus, SE plants flowered earlier than usual for the late-flowering morph (at week 8-9 *vs.* 18-21 weeks reported by Moser et al. 2009), although still about two weeks later than the DE plants. As expected, early-flowering

DE plants had longer stems at flowering, reached lower final size (stem biomass) and tended to produce less fruits and seeds. Thus, although our results are preliminary and need to be confirmed with further investigations including more populations of the two flowering types and more families within populations, they suggest that the genetic background of the assayed plants and even variance between individuals of the same family can affect the responses to experimental treatments (see also Herman and Sultan, 2016). In the following paragraphs, we discuss the main observed effects of the two treatments on the two provenances and further steps required to better interpret their contrasting responses.

EFFECTS OF SEED DEMETHYLATION TREATMENT

Use of 5-azacytidine in earlier studies showed impaired plant growth (see e.g., (Fieldes & Amyot, 1999; Finnegan et al., 1996; Kondo et al., 2006). Our 48 h treatment did not affect seed germination and was applied with a moderate concentration to avoid survival-related problems and any serious developmental effects (see also,(Akimoto et al., 2007; Bossdorf et al., 2010; Burn et al., 1993). Importantly, the treatment induced a moderate, statistically significant reduction in global DNA cytosine methylation only in plants from the early-flowering DE population and regardless of subsequent herbivory treatment. Such results were somehow unexpected according to previous studies conducted only in late-flowering plants (Burn et al., 1993). The novelty of this result stands in showing that the effect of seed-stage experimental demethylation can last to adulthood in short-lived plants, and not only in seedling leaf DNA as assessed previously (Alonso et al., 2017; Griffin et al., 2016; Puy et al., 2018). Also, the

magnitude of the effect varied with seed provenance and this might be due to small differences in permeability of seed coat and the physiological status of *T. arvense* seeds from different populations that would condition penetrance of 5-azacytidine during the short period of seeds imbibition we applied (see also Burn et al. 1993). Alternatively, we cannot discard that restoration of methylation marks in adult plants after 12-14 weeks since demethylation might change with provenance (Fieldes & Amyot, 1999; Kumpatla & Hall, 1998). Advanced methylome analyses based on deep sequencing after bisulfite conversion (see e.g., (Becker et al., 2011; Colicchio et al., 2018) would be required to fully understand the observed differences, e.g. a null change in global methylation can arise from similar frequency of additions and drops of methyl groups to cytosines in different genomic locations.

Immediately after demethylation, time to first leaf appearance delayed similarly for azacytidine treated plants from the two plant-types supporting that the treatment indeed altered the initial development in all of them (see also Burn et al., 1993; Finnegan et al., 1996; Kondo et al., 2006). The phenotypic response of the two populations to azacytidine treatment diverged after 7-8 weeks shortly after vernalization. Azacytidine reduced early growth more strongly in early-flowering DE plants that were shorter than their control relatives immediately before herbivory treatments started. However, at flowering onset, the effect of azacytidine did not significantly reduce plant height in the late-flowering SE plants, partially due to their different plant architecture of large rosette, but was still evident in early-flowering DE plants. Such finding suggests that late-flowering plants were able to recover faster from the initial delayed growth.

Our herbivory treatments were repeated twice to prime plants and elicit a stronger and fast defence response (Mauch-Mani et al., 2017; Sobral et al., 2021). However, we did not analyse the priming effect itself. We searched for the molecular systemic consequences in unwounded leaves collected after 24 h of the second event that could be detected as changes in DNA global methylation levels (Kellenberger et al., 2016), increased glucosinolate concentration (Textor & Gershenzon, 2009) or both. The herbivory simulation treatment included JA-spraying, a plant hormone involved in regulation of plant growth and defence (Züst & Agrawal, 2017) whose exogenous application is able to increase glucosinolates concentration in Brassicaceae plants and reduce subsequent insect consumption (Textor & Gershenzon, 2009; Fritz et al., 2010; Kellenberger et al., 2016; Jeschke et al., 2017). Accordingly, our artificial herbivory treatment increased sinigrin concentration. Further, it decreased final size (stem biomass) of late-flowering SE plants supporting a negative impact on plant performance that has not been frequently reported in studies that used just hormone application in other Brassicaceae species (Van Dam et al., 2004). This indicates that JA application together with a mild defoliation, induced the jasmonate cascade and changed the growth–defense prioritization more strongly than *P. brassicae* consumption in late-flowering SE plants (Züst & Agrawal, 2017). In regards to reproductive output, previous studies reported larger effects on seed mass and seed production after mechanical leaf defoliation conducted at flowering time compared to earlier and later treatments (Akiyama &

Ågren, 2012; Barto & Cipollini, 2005). In our study, artificial herbivory did not alter fruit or seed number likely due to lower defoliated surface and performance at an earlier developmental stage than the referred studies.

Interestingly the magnitude of the effects of insect and artificial herbivory on leaf DNA methylation and glucosinolate concentration, and their interaction with previous seed demethylation treatment varied with plant-type and were not significant in the early-flowering type. In the late-flowering SE population, that did not show differences in global DNA cytosine methylation after AZA treatment, DNA global methylation level of artificially damaged and insect-consumed plants were reduced significantly compared to undamaged control plants. Furthermore, an overall upsurge of glucosinolates was observed after herbivory in this population, although the effect was somehow herbivore-type specific and varied with demethylation treatment. Sinigrin, the most abundant aliphatic glucosinolate, increased more in artificially damaged plants, whereas insect eaten plants got higher concentration of indole glucosinolates such as 4-methoxyglucobrassicin, when treated with AZA at seed-stage. Furthermore, seed stage demethylated individuals undergoing artificial herbivory treatment had the highest sinigrin concentration, suggesting that DNA demethylation can regulate the jasmonate signaling cascade towards sinigrin biosynthesis (Textor & Gershenzon, 2009). This is relevant for commercial purposes because sinigrin is a precursor of mustard oil glucoside in seeds of *T. arvense* (Warwick et al., 2002) and we found surge in sinigrin after demethylation and artificial herbivory is better predicted for late-flowering SE plants. In the early-flowering DE

plants, global DNA methylation did not differ between any of the two herbivory treatments and controls. On average, artificial but not insect herbivory led to higher leaf glucosinolates concentration, despite the observed large variance among treated individuals from this population. Again, advanced methylome analyses could help to elucidate whether the observed population specific responses are mainly due to genetic divergence (see e.g., (Aller et al., 2018) for glucosinolate production), and/or epigenetic variation among them (see e.g., Latzel et al., 2012 for response to JA).

As regards plant performance, previous herbivory studies on the Brassicaceae family have showed contrasting results for artificial defoliation, hormone application and insect herbivore damage (Agrawal, 2000a; Tucker & Avila-Sakar, 2010; Sotelo et al., 2014; Kellenberger et al., 2016). Some earlier studies also showed reduction in seed production when insects consume leaf of young plants and suggested that tolerance levels increase from earlier to later developmental stages in crops and Brassicaceae plants (Boege et al., 2007; Tucker & Avila-Sakar, 2010; Sobral et al., 2021). Here, we have found contrasting responses to herbivory between the two study plant-types, similar to previous studies conducted with several accessions or provenances in other Brassicaceae (Manzaneda et al., 2010; Tucker & Avila-Sakar, 2010). Although further studies with more populations and families are needed before more robust conclusions can be drawn, our findings suggest that the two flowering ecotypes of *T. arvense* may have evolved different anti-herbivore strategies. We hypothesize that the early-flowering ecotype, that has a fast-growing cycle, may be more tolerant against herbivores since after damage plants did not alter their

chemical defenses but tended to increase reproductive output. Whereas, plants of the late-flowering ecotype, that need much more time to complete its growing-cycle, could be more resistant and less tolerant to herbivory after damage as they tended to invest more resources in increasing their chemical defenses while reducing seriously their growth and reproduction.

In our study, we can speculate also that despite being conducted on the same dates, both the priming and herbivory treatments reached plants at different stage of their life-cycle, more close to bolting in early-flowering DE type, and that could reduce the impact of insect herbivory (see Sobral et al., 2021 for an analysis of age effect). Such finding emphasizes the relevance of using multiple provenances to gain generalization in understanding plant responses to herbivory, and the lack of studies addressing so. Moreover, seed-stage demethylation treatment altered fruit production and seed mass after herbivory suggesting that the two treatments had contrary effects on the two plant-types. In particular, late-flowering SE plants that were azacytidine treated produced significantly less number of seeds per fruit and smaller seeds. In early-flowering DE, plants treated with 5-azacytidine at seed-stage tend to produce more fruits, more seeds per fruit, and heavier seeds and some of the differences become even larger in plants experiencing herbivory. As long as late-flowering winter type of *T. arvense* is currently emerging as a new winter biofuel crop (Sedbrook et al., 2014; Dorn et al., 2015, 2018; García Navarrete et al., 2022), our findings could be relevant somehow for future research towards improvement of seed yield and reduction of glucosinolate content in potential new crop varieties.

CONCLUSION

Overall, this study illustrates the importance of DNA methylation variation in plant performance and short-term chemical defence after herbivory, supports experimental demethylation as a useful approach to investigate epigenetic regulation of plant-herbivory interactions and reveals the value of including different modes of herbivory and plant provenances to avoid oversimplification. According to our initial predictions we can conclude: i. Seed-stage demethylation is suitable to alter DNA methylation levels in leaves of reproductive adult plants of *T. arvense,* although the magnitude of the effect can vary between populations of origin and flowering ecotypes. A longer treatment could perhaps produce stronger effects. ii. Herbivory had different effects depending on provenance. In SE late-flowering plants it increased glucosinolates concentration, and reduced final size and fruit production. However, the effects were non-significant in DE early-flowering type except for seed production. iii. Herbivory reduced DNA methylation only in plants of late-flowering SE type, the effect being stronger for the artificial treatment. iv. For early-flowering DE type demethylation increased reproductive output mainly in plants experiencing herbivory somehow reducing its detrimental effect (i.e., increased tolerance). Such effect was not observed, however, in late-flowering plants, in which the two treatments reduced reproductive output. Altogether, such findings indicated that variation in DNA methylation had subtle interactions with plant response to short-term herbivory and the responses depend largely on plant ecotype associated to geographic origin, genetic background and the life-cycle phenology. Deeper methylome and transcriptome analyses need to be conducted

for a more comprehensive understanding of molecular epigenetic mechanisms that regulate plant responses to herbivory.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

A.N.T. designed and performed the experiment, analyzed the data and drafted the paper; C.A. and M.M. conceived and designed the experiments, contributed to experiment development, and sample analyses; C.M. provided the glucosinolate data and suggestions on analysis; all authors contributed to refine writing.

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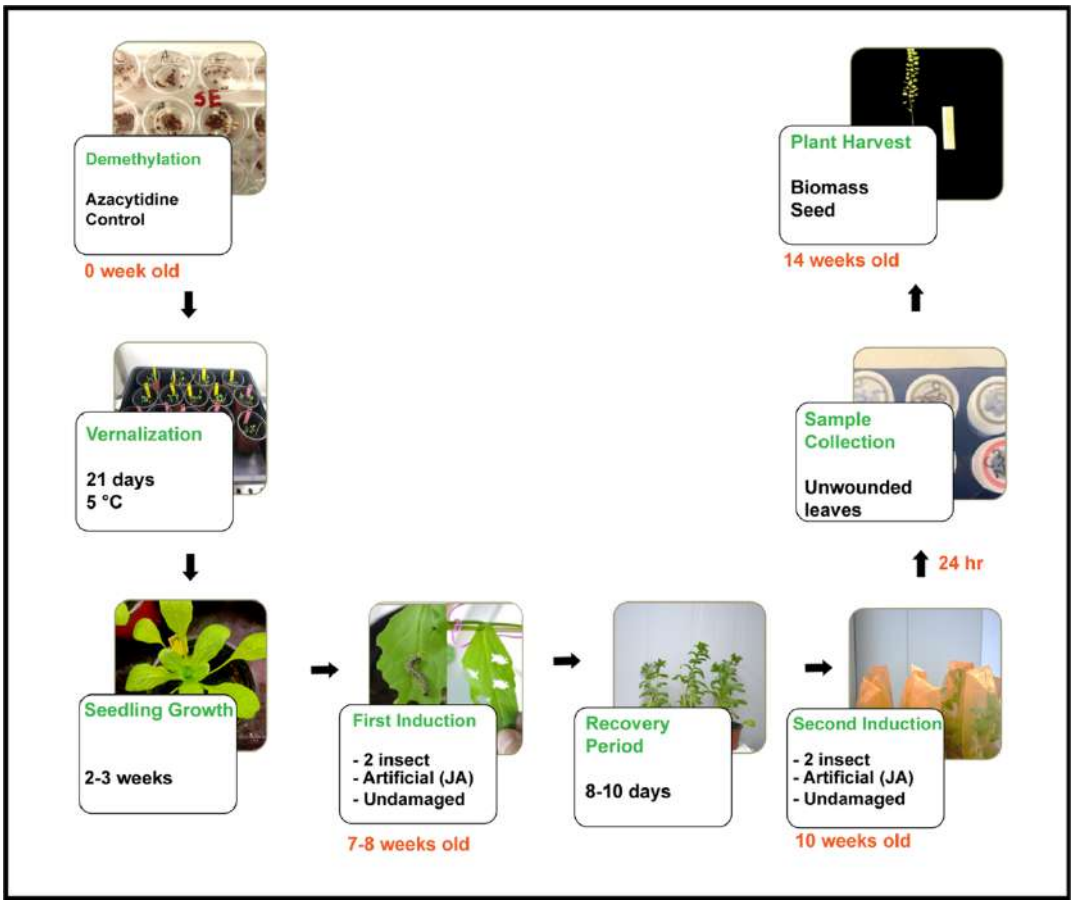
SUPPLEMENTARY MATERIAL

Supplementary Figure S1. Phenotypic features of typical individual adult plants from the two provenances at the time of harvesting. (A) a late-flowering SE plant with short internodes within the flowering/fruiting shoot, note that still some fruits were green; (B) an early-flowering DE plant with longer internodes within the flowering/fruiting shoot that initiates from the bottom-most section, note that all fruits were dried.

Supplementary Figure S2. Experimental timeline indicating treatments, vernalization, sampling and harvesting of *Thlaspi arvense*



Supplementary Table S1

Table S1 Summary of the ANOVA results of the full factorial design conducted in a growth chamber to analyze the effects of demethylation, herbivory and their interaction on global DNA cytosine methylation, final stem biomass, total fruit number, average seed number per fruit, average seed mass and leaf glucosinolates in plants of *Thlaspi arvense* from Swedish (late-flowering SE) and German (early-flowering DE) populations. The design included 2-4 replicates per family and family was included in the model as a random effect. Significant effects are shown in bold.

Response variable	*Source of Variation*	***Late-flowering SE***			***Early-flowering DE***		
		χ^2	**DF**	**P-value**	**χ^2**	**DF**	**P-value**
Global DNA cytosine methylation	Demethylation	0.02	1	0.877	3.48	1	0.062.
	Herbivory	6.52	2	**0.038**	0.25	2	0.881
	Demet x Herb	0.06	2	0.969	0.86	2	0.650
Final Stem Biomass (g)	Demethylation	0.001	1	0.937	1.07	1	0.301
	Herbivory	10.25	2	**0.005**	4.13	2	0.126
	Demet x Herb	1.16	2	0.560	2.03	2	0.362
Total Fruit number	Demethylation	0.03	1	0.872	9.56	1	**<0.001**
	Herbivory	13.36	2	**<0.001**	5.89	2	**0.052**
	Demet x Herb	3.97	2	0.137	1.04	2	0.595
Average Seed number per fruit	Demethylation	7.45	1	**0.006**	12.45	1	**<0.001**
	Herbivory	1.03	2	0.597	7.88	2	**0.020**
	Demet x Herb	1.56	2	0.459	2.27	2	0.321

Average seed mass (mg)	Demethylation	3.88	1	**0.048**	10.81	1	**<0.001**
	Herbivory	1.69	2	0.431	2.19	2	0.335
	Demet x Herb	0.68	2	0.712	5.62	2	0.060.
Total GLS (µmol.g−1 d.w.)	Demethylation	5.14	1	**0.023**	0.06	1	0.801
	Herbivory	40.37	2	**<0.0001**	4.73	2	0.094.
	Demet x Herb	7.73	2	**0.02**	0.34	2	0.847
Sinigrin (µmol.g−1 d.w.)	Demethylation	5.06	1	**0.02**	0.06	1	0.801
	Herbivory	38.95	2	**<0.0001**	4.72	2	0.094
	Demet x Herb	7.77	2	**0.020**	0.33	2	0.847
4-methoxyglucobrassicin (µmol.g−1 d.w.)	Demethylation	0.2	1	0.655	0.37	1	0.541
	Herbivory	6.2	2	**0.04**	0.61	2	0.739
	Demet x Herb	6.18	2	**0.04**	2.03	2	0.363

CHAPTER 3: EPIGENOMIC RESPONSE TO INSECT HERBIVORY IN LOMBARDY POPLAR: ASSESSING GEOGRAPHIC VARIANCE IN MODIFICATION OF DNA METHYLATION

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ABSTRACT

As a key epigenetic mechanism in plants, DNA methylation is responsive to environmental cues and may contribute to tailor individual responses to stress, being particularly beneficial for long-lived trees and clonal plants. The risk for insect outbreaks is predicted to increase in temperate areas, thus, understanding to what extent changes in DNA methylation can help forest trees to develop locally adapted responses across different geographical backgrounds could be particularly useful for assessing the fate of trees and forests under global change. Here, we study the responses of the cultivar *italica* of the long-lived woody tree *Populus nigra* (Lombardy poplar), a fast-growing variety that has been asexually propagated (by division) with economic purposes. Cuttings were collected from three distant geographical locations (Spain, Italy, Poland), clonally replicated and grown in a common environment. We used reduced representation bisulfite sequencing, epiGBS, to assessing changes between three plant categories: (i) insect herbivory, (ii) artificial herbivory and (iii) undamaged plants, across populations. Our quantitative results indicate that genome-wide DNA methylation in Lombardy poplars from different geographical provenance responded differently to experimental herbivory, with origin of plants accounting for 17.2 % of the variation in DNA methylation across CG, CHG, and CHH contexts. Both insect and artificial herbivory induced a significantly higher response in the CHH context, suggesting that CHH methylation was the most plastic. When we pay attention to those loci with contrasting methylation levels (Differentially Methylated Cytosines, DMCs) between undamaged plants and the two types of herbivory assayed we found some mixed results: Spanish ramets were most responsive to insect herbivory, Polish ramets to artificial herbivory, and Italian ramets were the least responsive in both cases.

Within the genome, DMCs were primarily located in gene bodies and their flanking regions (CG, CHG) or intergenic regions (CHH). Further, about one-third of DMCs obtained in CHH context were associated with transposable elements (TEs), suggesting a strong association between changes in non-CG methylation and TEs in response to leaf damage. Support for a geographically variable DNA methylation in response to herbivory in a clonal tree with reduced genetic variation highlight the importance of epigenetic mechanisms in shaping both tree plantations and natural forests to their environmental challenges.

***Keywords*:** bisulfite sequencing, clonal propagation, differentially methylated cytosine (DMC), DNA cytosine methylation, forestry, insect herbivory, jasmonic acid, *Populus nigra* cv. 'italica', transposable elements, tree provenance.

INTRODUCTION

Forests occupy large areas in Europe, where canopy trees provide food and shelter for numerous animals, fungi, and microorganisms; act as valuable understory habitats for a diverse community of plants; are key actors in interspecific interactions; and provide multiple ecosystem services. (See e.g., Basile et al., 2020; Eberl et al., 2020). Climate change is predicted to impact on European forests but the magnitude of such effects will vary geographically, because bioclimatic zones in Europe differ both in their limitations for tree growth and in the expected shifts in temperature and drought risk (Intergovernmental Panel On Climate Change (IPCC), 2023; Lindner et al., 2010). Changes in climate are also causing shifts in the

distribution range of insect species and the risk for insect outbreaks is predicted to increase (Lindner et al., 2010; Bebber, 2015; Canelles et al., 2021). The consequences of those outbreaks will depend on both intrinsic and extrinsic factors. For instance, it is well-established that some plants are able to change leaf quality in response to herbivory (e.g., Havill & Raffa, 1999) and, undergoing both within-generation plasticity and/or transgenerational defence induction. That in turn, improve either growth or defence against herbivory and thus being able to at least in part buffer the consequences of defoliation (Holeski, 2007; Holeski et al., 2012; Holeski et al., 2013). Understanding the mechanisms underlying the induced response to herbivory in trees, with their characteristically longer life spans, and how they may vary geographically could be particularly useful for assessing the fate of trees and forests under global change (Aitken et al., 2008).

Epigenetic regulation has emerged as a significant element in plant adaptation to heterogeneous environments and stress response (Richards et al., 2017). In particular, DNA cytosine methylation is an epigenetic mark that is involved in determining to what extent plants can modify their features in response to biotic and abiotic stresses (Chang et al., 2020; Ramos-Cruz et al., 2021; Zhang et al., 2018). In plant genomes, DNA cytosine methylation occurs in three sequence contexts (CG, CHG, and CHH, where H = A, C, or T), each one maintained or established de novo through different enzymatic pathways, and featuring distinct genomic locations, inheritance patterns and reversibility (Hofmeister et al. 2020; Zhang et al., 2018). Symmetric methylation in the CG sequence context is maintained during DNA replication because hemi-methylated CG sites are recognized by enzymes that catalyze CG methylation on the newly synthesized strand by way of 'template copying', it is often located in gene bodies (gbM), and it has

been reported to be stable across generations (Dubin et al., 2015; Niederhuth et al., 2016; Yao, Schmitz, et al., 2021). In *Arabidopsis*, gbM is typically found in moderately and constitutively expressed housekeeping genes, although its biological significance in other species is still uncertain (Niederhuth et al., 2016; Muyle et al., 2022). DNA methylation in the CHG and CHH sequence contexts is preferentially targeted by *de novo* methylation pathways —with CHH being less stable along cell division, it is predominantly found in repetitive sequences and transposable elements (TEs), which are more abundant in species with larger genomes (Zemach et al., 2010; Bewick & Schmitz, 2017a; Kenchanmane Raju et al., 2019; Ramakrishnan et al., 2021a; Yao, Zhang, et al., 2021). It is important to remark that spontaneous methylation gains and losses at individual cytosine positions in any sequence context occur at rates four to five orders of magnitude higher than genetic point mutations per unit time, whereas inheritance of such spontaneous methylation changes is mainly restricted to CG sites and to some extent to CHG (Yao et al., 2021). Thus, in long-lived plants divergence in methylation profiles can occur within individual plants (Herrera et al., 2021; Hofmeister et al., 2020; Yao et al., 2021).

Previous studies have demonstrated that individual plants can have functional stress memory, that is, pre-exposure to a certain stress generates a more pronounced and/or faster response against subsequent events, and that defence priming in response to herbivory is linked to epigenetic processes (Kim & Felton, 2013; Mauch-Mani et al., 2017). For example, in *Thlaspi arvense*, plants that have been primed and elicited a second herbivory event exhibited changes in terms of chemical defense and global DNA methylation levels compared to undamaged plants, and the magnitude of change varied between two ecotypes (see Chapter 2 of this thesis and Troyee et al., 2022).

A genotype specific phenotypic response to insect herbivory and artificial demethylation in *Brassica rapa* further substantiates the complexity of DNA methylation changes after insect herbivory (Kellenberger et al., 2016). Transgenerational effects of herbivory, where the offspring of damaged plants have altered levels of defence relative to the offspring of control plants, have been also related to epigenetic changes. For instance, Rasmann et al. (2012) showed that *Arabidopsis* and tomato plants with insect-damaged parents exhibited significantly enhanced resistance against herbivory by the same insect species. More interestingly, *Arabidopsis* mutants defective in the RNA-directed DNA methylation (RdDM) pathway did not show the inheritance of resistance, indicating involvement of DNA methylation in transgenerational priming of defence. Also, in *Mimulus guttatus* parental damage produces a strong and consistent defensive response, increasing trichome production, that varies geographically according to natural levels of herbivory (Akkerman et al., 2016), it is transmitted directly to the progeny and seems to be strongly associated with the inheritance of altered methylation profiles and differentially expressed genes (Colicchio et al., 2018; Monnahan et al., 2021). Moreover, both the sequence context and genomic location of stress-induced methylation changes seems to be relevant in developing a response, likely related to the contrasting patterns of inheritance of spontaneous methylation changes mentioned above. For instance, interactions between methylation in promoters and gbM at CG and CHG are relevant to understand gene expression in *Mimulus* (Colicchio et al., 2015) but also heritable DNA hypomethylation at selected TE-rich regions causes genome-wide priming of defence genes and high levels of disease resistance in *Arabidopsis* (Cooper & Ton, 2022).

Epigenetic mechanisms should be particularly valuable for long-living organisms such as trees although current knowledge on their contribution to herbivory is still scarce (Bräutigam et al., 2013). Also, as mentioned above, most of our current knowledge on the relationships between epigenetic marks and plant response to herbivory refers to annual model plants and crops (Rasmann et al., 2012; Kim & Felton, 2013; Kellenberger et al., 2016; Colicchio et al., 2018). In this study, we analyse DNA methylation changes in response to herbivory in leaves of the Lombardy poplar (*Populus nigra* cv. 'italica' Duroi). Our study species, is a cultivated variety of the fast-growing tree *Populus nigra* L., originated from a single male mutant that has been propagated by artificial vegetative reproduction (cuttings) through Europe and worldwide since the beginning of eighteen century (Elwes & Henry, 1913). These characteristics, namely clonal propagation and widespread distribution, make the species ideal to study the epigenetic mechanisms involved in response to herbivory for several reasons. First, European populations exhibit low levels of genetic variation (Díez Rodríguez et al., 2022a), thus, it is more likely that Lombardy poplar trees would rely on epigenetic mechanisms to adapt and respond to geographic differences in environmental challenges (Vanden Broeck et al., 2018). Second, previous studies in poplars have demonstrated the induction of defences after herbivory (Havill & Raffa, 1999; Mccormick et al., 2014) and distinct transcriptional responses to insect and artificial herbivory (Major & Constabel, 2006; Babst et al., 2009; Philippe et al., 2010; Müller et al., 2019). Finally, its wide distribution allows us to study whether the methylation changes in response to herbivory may vary across geographic regions with contrasting environmental conditions, provided that lineage-specific and

environmental-driven epigenetic marks contribute to define locally adapted ecotypes (Vanden Broeck et al., 2018).

Here, we investigate DNA methylation variation in response to herbivory in clones of *P. nigra* cv. 'italica' from three geographically distant provenances with contrasting environmental conditions (Spain, Italy and Poland). We applied two herbivory treatments, including true consumption by caterpillars of *Lymantria dispar* (Lepidoptera: Erebidae), and artificial leaf damage conducted by manual wounding combined with jasmonic acid spraying. We compared these treatments with undamaged control plants using epiGBS, a reduced representation bisulfite sequencing tool that is able to identify the sequence context and genomic location of DNA methylation at single base resolution (Gawehns et al., 2022; Troyee et al., 2023 and Chapter 4 in this thesis). Our main objective was to elucidate if and how DNA methylation responds to herbivory, with a focus on discerning common and distinct epigenetic alterations induced by the two herbivory treatments, and if the response depends on the geographical (environmental) origin of the tree. We tested the following specific questions: (1) Does genome-wide DNA methylation of Lombardy poplars originated from contrasting geographic provenances respond differently to herbivory? (2) Do insect and artificial herbivory induce comparable methylation changes? (3) Where within the genome are herbivory-induced DNA methylation variations most prevalent?

MATERIALS AND METHODS

EXPERIMENTAL PROCEDURE

Plant Materials and Growth Conditions

Cuttings of 2-3 field growing adult trees from each of three distant European populations of *P. nigra* cv. 'italica' with contrasting climates (see graphical abstract at General Introduction in this thesis) were collected in spring 2018 and transplanted into a common garden in the Marburg Botanical Garden (Germany; 50º 48' 02.7" N, 8º 48' 24.8" E) for 10 months (Díez-Rodríguez et al., 2022b). These three natural populations were located in the north of Spain (41°31'45.7"N 4°42'22.1"W), the north of Italy (44°35'22.0"N 11°03'25.8"E), and the center of Poland (52°40'17 "N 19°04'16 "E). From each of the garden grown trees ("ortet") we selected and cut six to nine similar sized branches (ramets herafter), that were ~30 cm long and ~15 weeks old (N = 63 ramets in total). Therefore, "ortets" are the garden grown trees originated from cuttings collected in the field (F0), and "ramets" are the clonally generated descendants (F1) that we use in the current herbivory experiment. Ramets were stored in the dark at 4°C for two weeks, afterwards they were soaked overnight in a rooting solution (50 mg/L Rhizopon AA 50 mg tablets) and planted in 2L pots, three per pot, containing a 1:1 sand:peat mixture (30% coarse sand, 20% fine sand, and 50% nutrient-poor potting soil) and 5 ml of rooting solution. Two weeks later, rooted cuttings were transplanted into individual 2L pots with the same 1:1 sand:peat mixture and regularly watered every three days. The slow-release fertilizer Osmocote Exact Mini (16+8+11+2MgO+TE) was added to each pot, three grams per pot, two weeks before the start of the experiment. The greenhouse conditions

during the experiment were as follows: temperature (day/night) 22/18°C (±2°C), relative humidity: 60% (±5%), light (day/night): 16/8 h. Each of the experimental levels of treatment included three ramets per ortet. To avoid volatile organic compound (VOC) exchange, we did not group plants from various treatments together (Mccormick et al., 2014; Moreira et al., 2016). The ramets assigned to each treatment level were split in two trays (flood tables), randomized within the tray (once per week) and watered regularly every three days.

Herbivory Treatments

We employed two herbivory treatments, including true consumption by *Lymantria dispar* (Lepidoptera: Erebidae) caterpillars and artificial leaf damage conducted by simulated wounding combined with jasmonic acid (JA)-spraying, and compared them with undamaged control plants. *Lymantria dispar* is a highly polyphagous herbivore that can cause severe damage in European mixed forests (Boukouvala et al., 2022). Mechanical wounding with addition of JA and *L. dispar* consumption were expected to elicit significant responses, and by using simulated herbivory we gained precision in the application of damage and control over the introduction of material from foreign and unidentified organisms (e.g., pathogens) by live insects (Havill & Raffa, 1999; Züst & Agrawal, 2017; Waterman et al., 2019).

To control for potential positional effects, damage was always inflicted on leaves of the lower half of the main branch of each ramet, and methylation analyses were done in tissue taken from the most adjacent undamaged leaves grown in the branch's apical half (see graphical abstract at General Introduction in this thesis). Both insect and artificial herbivory treatments were performed twice in order to induce a priming effect (first

treatment) and elicit a stronger and/or quicker response during the second treatment (Mauch-Mani et al., 2017). A more detailed description of the experimental procedure and set up can be found at Chapter 4 in this thesis (see also General Introduction and Troyee et al., 2023). For insect herbivory, the plants were primed with ten *L. dispar* L2 caterpillar larvae that were placed on the fully expanded leaves of the main branch of our experimental poplar ramets, which were encaged in 75*100 cm nylon mesh bags. After five days, the larvae were removed from the plants for three days to allow them to recover. For the second insect herbivory event, seven L2 instar and five L4 instar larvae were placed on lower main branch leaves, encaged in the same nylon mesh bag, and allowed to feed freely for seven days prior to the collection of leaf samples (see below).

Artificial herbivory was also conducted in the main branch and in similar locations than insects were encaged. In the priming phase, three to four leaves of the lower half of the main branch were punched with 6 to 8 holes (approximately 3 to 5 mm in diameter) per leaf. Immediately following the artificial wounding, two pumps (150 µL per pump) of a JA solution were sprayed onto the damaged leaf and three pumps were sprayed throughout the plant. This procedure was repeated three days later for the second artificial herbivory treatment by punching a total of 10-12 punched leaves per ramet.

In the control group, similarly positioned, well-developed leaves from each ramet's main branch were sprayed with an equivalent aqueous solution containing no JA and covered with nylon mesh bags in the same manner as the herbivory treated leaves. The experiment was concluded 17 weeks after clonal propagation.

Sample Collection

The tissue of undamaged and completely expanded leaves of the adjacent apical half of each ramet was collected 24 hours after the second herbivory event in treated plants, or after the aqueous spraying in controls, seeking to characterize systemic changes (*sensu* Babst et al., 2009). Throughout the duration of the experiment, these leaves were left unbagged. Five-6 discs of leaf tissue (approximately 3-5 mm in diameter) were removed using a cork borer and promptly frozen in liquid nitrogen. The vials were stored at -80 ºC until DNA extraction. The order of sampling and DNA extraction was determined by randomly selecting one sample per treatment (regardless of ortet) at a time. Frozen leaf material was grinded and homogenized using a Qiagen TissueLyser II equipped with two stainless steel balls (45 seconds at a frequency of 30.00 1/s). The NucleoSpin Plant II reagent from Macherey-Nagel was used for DNA extraction, and cell lysis Buffer PL1 (CTAB method) was used to obtain the highest possible DNA quality.

LIBRARY PREPARATION AND SEQUENCING

With a few adjustments, we followed the new EpiGBS2 optimized protocol (Gawehns et al., 2022). First, the samples were randomized, and 1000 ng of DNA from each sample were digested with the restriction enzymes AseI and NsiI. The digested DNA was then ligated with hemi-methylated adapter pairs encoding barcodes of sample-specific 4-6 nucleotides. These barcodes also included three random nucleotides (NNN), used during bioinformatic analysis to identify PCR duplicates as well as an unmethylated cytosine, used to annotate the Watson and Crick strands and in order to determine the bisulfite conversion rate. The samples were then multiplexed, concentrated,

and smaller fragments (60 bp) removed using the NucleoSpin Gel & PCR cleaning Kit. SPRIselect™ magnetic beads (Beckman Coulter™) were used to select 300 bp DNA fragments (and lower). To get fully ligated and methylated adapters, deoxynucleoside triphosphates (dNTPs) containing 5-methylcytosine were used to repair the nicks caused by hemi-methylated adapters. We followed the protocol from the EZ DNA Methylation-Lightening kits to convert the unmethylated cytosines in the DNA to uracils, which were then substituted by thymines during the amplification stage. The transformed DNA was PCR-amplified, followed by a final clean-up and size selection. The library was then paired-end (PE 2x150bp) sequenced in one lane of an Illumina HiSeq 4000 sequencer with a 12% phiX spike.

epiGBS Data Processing

The epiGBS2 pipeline's 'reference' branch was used to process the sequencing data (Gawehns et al., 2022). All steps were implemented into a Snakemake 6.1.1 workflow (Köster & Rahmann, 2012). First, PCR duplicates were removed based on the 3-random nucleotide sequence (NNN) inserted into the adapter sequences. The purpose of this step was to confirm true PCR clones so that they could be removed from the sequencing data, but not biological duplicates. Using Stacks2 software (Catchen et al., 2013; Rochette et al., 2019) the samples were demultiplexed according to the barcodes, followed by adapter removal using cutadapt (Martin, 2011). Our 63-sample library generated 740,184,096 raw sequencing reads, of which 475,316,207 (64.2%) were successfully demultiplexed and assigned to individual samples. These data are stored in ENA projects: PRJEB51623 and PRJEB51853. The bisulfite conversion rate (94.9 %) was calculated by estimating the number of correctly bisulfite-converted control cytosines within the adapters (see

Gawhens et al., 2022). Next, the pipeline mapped the sequencing reads of experimental data to the reference genome of *P. nigra* cv. 'italica' (available at ENA project: PRJEB44889) using the default parameters of BISMARK v0.19.0 (Krueger & Andrews, 2011). Finally, strand-specific methylation and nucleotide calling were performed within the pipeline to obtain one single methylation polymorphism bed file per sample (containing rows with chromosome/scaffold name, genomic position, strand information, methylated cytosine number, unmethylated cytosine number, cytosine context (CG/CHG/CHH), and trinucleotide context information where H are indicated by true A, T, C in the sequence).

Data Quality Filtering

First, we filtered methylation data by removing the samples with low read coverage, that is, samples that had less than 50 % of the average number of cytosines present across samples. All 63 samples passed this filtering. Next, cytosine positions covered with less than 10 reads (10x) were removed and resulting files for each sample stored as flat file databases in R package methylKit (Akalin et al., 2012). The coverage of the Cs per sample was normalized using the *normalizeCoverage* function (method = "median") in methylKit. For each population, individual databases were then merged using the *unite* function of methylKit including all Cs present (with minimum 10x coverage) in at least 2 out of 3 of the samples per treatment group. The final methylation call dataset contained a total of 5,717,923 cytosine reads (1,630,832, 2,207,164, and 1,879,927 for Spain, Italy and Poland respectively). As the presence of C > T SNPs can impact the accuracy of detected methylation levels, we also excluded 5,691 Cs that overlapped with SNP positions that were present in the epiGBS SNP file. After removing cytosines

with at least one missing value across all samples, we obtained 108,724 positions (1.9 % of total) without any missing value. The overall multivariate analyses were performed on this dataset (see below).

EPIGENETIC ANALYSES

All the following statistical analyses were carried out using the R environment (R Core Team, 2022). At every sequenced cytosine locus in a sample, methylation level (%) was calculated as: (methylated cytosine read count)/ (methylated cytosine read count + unmethylated cytosine read count) * 100. Likewise, for each sample, we obtained genome-wide methylation levels as the average methylation levels of all cytosine loci sequenced for that sample. Given that in plants DNA methylation can occur within three cytosine contexts (CG, CHG, CHH) and that on each context has distinct properties and functions, statistical analyses were conducted also for each context separately (see details below).

Multilocus Redundancy Analysis (RDA)

To asses and quantify the contribution of population of origin and herbivory treatment to the variation in cytosine methylation levels, we used a multivariate method called redundancy analysis (RDA) as implemented in the package *vegan* 2.6 using the function *rda* (Oksanen et al., 2022). RDA is a constrained ordination method analogous to linear regression for cases that have multiple-dependent variables (in our case, DNA methylation values for each cytosine site) and several independent variables (in our case, population of origin and herbivory treatment). The significance of the models was tested using the function *anova ()* of the package stats that uses type-II sum of squares. These analyses were conducted using as the response matrix the

DNA methylation data shared by all the samples of the three populations, i.e. without any missing values, that had 108,724 cytosine positions (11,462 in CG; 17,043 in CHG; and 80,219 in CHH).

First, we assessed to what extent variation in methylation levels among samples can be explained by differences between populations and ortets using only the group of control samples (N = 21). We run two different models that allowed us to test for (i) epigenetic differentiation among populations, and (ii) differentiation among ortets after adjusting for the variance explained by the populations, as follows:

i) DNA methylation matrix ~ population;
ii) DNA methylation matrix ~ ortet + Condition (population).

Next, to assess the effect of herbivory on DNA methylation variation and test whether this effect differed among populations, we ran the following model for each herbivory type separately (control *vs.* insect herbivory and control *vs.* artificial herbivory) in order to detect a potential divergence in the strength of the methylation response elicited:

iii) DNA methylation matrix ~ population + herbivory

Differentially Methylated Cytosines (DMCs)

Since a large number of positions were not shared among the three study populations, differential methylation analysis in response to experimental treatments was conducted for each population separately by using the methylation call datasets, that included Cs with a minimum coverage of 10x and present in at least 2 out of 3 of the samples per treatment group (see above). On each population data, we searched for differentially methylated cytosines (DMCs) i.e., cytosines with a statistically significant minimum

methylation difference of 10 % between ramets assigned to any of the two herbivory groups in comparison to the control group. DMCs were called using a generalized linear model as implemented in the R package methylKit, including herbivory (insect or artificial) as the fixed factor and ortet as covariate. The method assumes that the methylated and unmethylated counts follow a binomial distribution and the effect of the fixed factor can be estimated with a log-likelihood test for logistic regression. MethylKit allows parameter adjustment to correcting by multiple testing based on *q*-value (*q*-value < 0.05), the minimum methylation difference (fixed = 10 %), and direction of methylation shift respect to control (hyper or hypo). We also looked for "stress-specific" DMCs, present in only one of the two herbivory treatments, and "non-specific" DMCs, i.e., those shared by insect and artificial herbivory treatments.

Finally, we annotated all the herbivory induced DMCs from the former analyses to different genomic features to explore the distribution of methylation changes across the genome. We overlapped DMC location with the *P. nigra* cv. "italica" genome (ENA project: PRJEB44889) that reports 41.7754.133 bp, and provides annotation for 40,988 gene models, with an average length of 3175.5 bp (including exons and introns) which collectively represent 31.15% of the sequenced genome (Dubay, 2024). We distinguished the following genomic features: i. the gene body, defined as the entire gene from the transcription start site to the transcription termination site, so that it includes exons and introns; ii. The promoter, defined as the region < 2 kb up-stream of the transcription start site, and iii. The downstream region, defined as the region located < 2 kb down-stream of the transcription termination site. DMCs located out of those three features were classified as intergenic regions that, after accounting for the average gene length and the

assigned fixed length of the two flanking regions, would be estimated as ca. 29.6% of the sequenced genome. We followed the approach used by Lin et al. (2022) to avoid assigning sites to multiple overlapping genomic features. We annotated sites following the order: gene body > 2 kb upstream of genes > 2 kb downstream of genes > intergenic regions, where a site annotated to a former feature would be excluded from subsequent annotation. In this order, if a site was annotated to "2 kb downstream of genes," it would not be annotated to the "intergenic region". Coordinates of each DMC were used to perform the BEDTools intersect command and a custom R script for annotating each genomic feature. To explore the methylation dynamics on Transposable Elements (TEs), we also searched for DMCs annotated within TEs. Transposable elements were annotated based on the TE prediction available in ZENODO (https://zenodo.org/deposit/7193978). Following the classification proposed by Peña-Pontón et al. (2024) based on TE predictions for *P. nigra* cv. *italica* reference genome, TEs were grouped into eleven of the most important superfamilies, namely Class I LTR (copia, gypsy, unknown, SINE), or retrotransposons, and Class II DNA transposons (DTA, DTC, DTH, DTM, DTT, Helitron, mite) that collectively represent ca. 34.8% of the sequenced genome (Dubay 2024). In particular, Class I LTR Gypsy elements accounted for the greatest proportion of projected TE (38%), followed by Class I LTR Copia (14%), Unknown Class I LTR (8%), Class II DNA/DTC (8%), Class II Helitron (7%) and Class II DTM (6%), other categories accounting for < 4% (Dubay, 2024). A cytosine was associated with a TE when it was located inside the TE, and only the shortest predicted TE was retained. If a DMC was annotated within a TE, hereafter, it will be referred to as a DMC-TE. Gene models and TE predictions used in this study were generated as part of the ongoing *P. nigra* cv. 'italica' reference genome

project (PRJEB44889). With the annotation result for each population, we developed Venn diagrams to illustrate to what extent DMCs and DMC-TEs were exclusively found in one population. Additionally, we ranked the DMCs and DMC-TEs and extracted the top 5% with highest hypo/hypermethylation difference between experimental groups and controls, which we termed as '*strongly responding*' DMCs.

RESULTS

Average DNA methylation level across the 63 study samples ranged between ~28.7-32.6 % in CG, ~15.1-17.8 % in CHG and ~7.4-8.2 % in CHH. Overall, genome-wide DNA methylation exhibited a right-skewed distribution, with many cytosines showing very low methylation levels (close to 0 %) and fewer showing high methylation levels (close to 100 %), except for CG methylation whose distribution was nearly bimodal (i.e., more cytosines with high methylation levels; Figure 1).

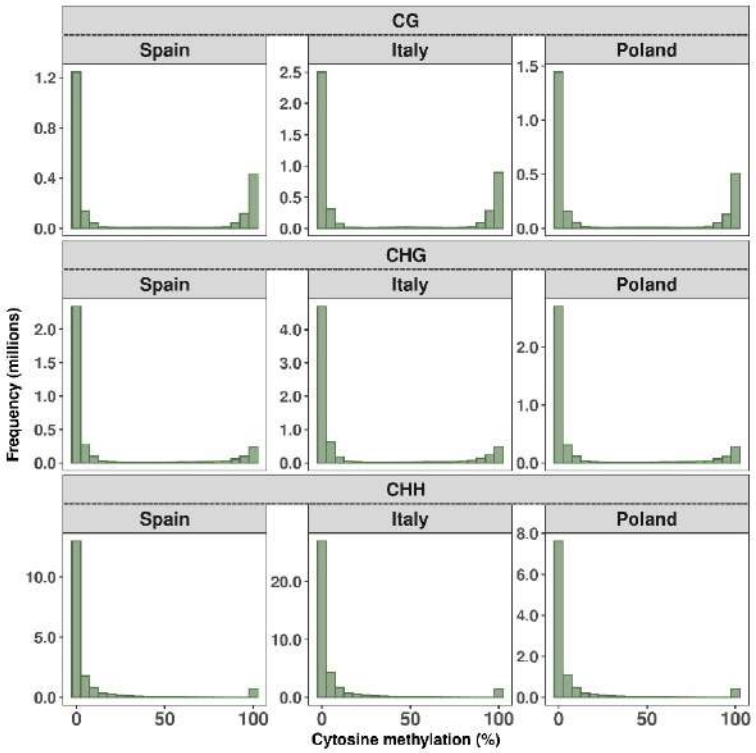

Figure 1. Frequency distribution of methylation levels per cytosine (%) in CG, CHG and CHH contexts across all the samples coming from the three studied populations (N = 18, 27 and 18 samples from Spain, Italy and Poland, respectively). Note that vertical axes have different scales according to the number of total Cs recorded for each context and population.

GENOME-WIDE DNA METHYLATION VARIATION AMONG POPULATIONS AND EXPERIMENTAL TREATMENTS

Figure 2 shows variation in the average genome-wide DNA methylation per context recorded across samples from the three study populations assigned to each of the three levels of herbivory treatment. The RDA analysis including only the control samples (N = 21) revealed that: i. population of origin significantly explained 17.2 % of the genome-wide methylation variation

across samples (F = 1.87, df = 2, P = 0.001); and ii. Ortet explained up to 29.5% of methylation variation (F = 1.94, df = 4, P = 0.001).

Moreover, the RDA analysis used to test for the effect of population and herbivory treatments on genome-wide DNA methylation showed that population explained a significant proportion of the variation in DNA methylation in all sequence contexts and both herbivory types (Table 1). Finally, only in the CHH context, herbivory explained a significant proportion of the DNA methylation variation in both insect herbivory and artificial herbivory samples (Table 1) with the methylation level of control plants being, on average, lower than in plants that experienced insect or artificial herbivory (Figure 2).

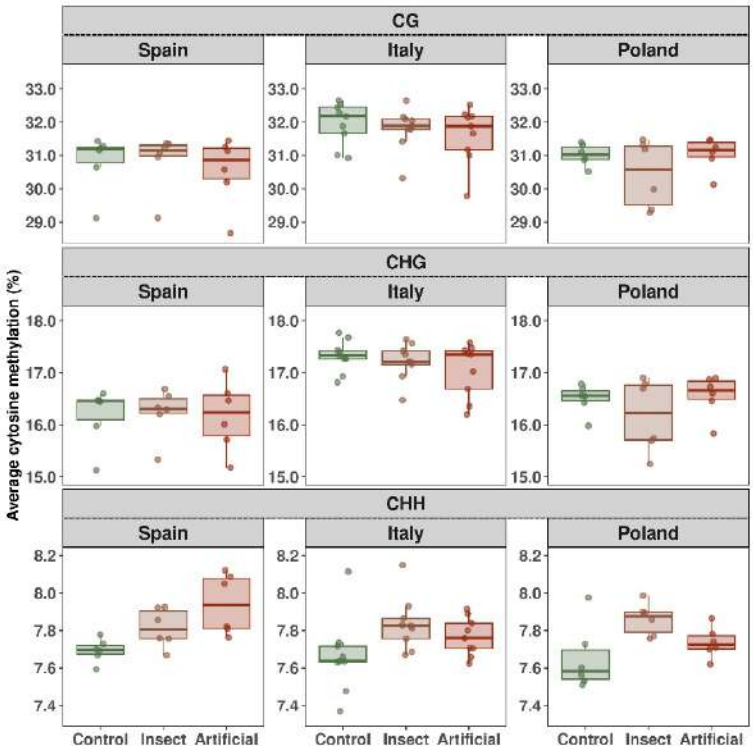

Figure 2. Box-plots showing variation in average genome-wide DNA cytosine methylation (%) between ramets from the three study populations assigned to each of the three levels of herbivory treatment (control, insect and artificial herbivory). Each dot represents a ramet (N = 18, 27, and 18 for Spain, Italy and Poland, respectively). Lower and upper box boundaries are the 25th and 75th percentiles, respectively, line inside box the median, lower and upper error lines the 10th and 90th percentiles.

Table 1. Results of redundancy analysis carried out to test for the effect of population of origin and herbivory treatment on genome-wide DNA methylation separately for the three contexts (CG, CHG, CHH) and for insect and artificial herbivory. The results of the permutation test (n = 999) for the significance of each of the predictors are shown. Values are in bold when $P \leq 0.05$.

Herbivory	*Context*	*Predictors*	*df*	*Variance*	*F*	*Pr (>F)*
Insect	CG	Population	2	97383	5.39	**0.001**
		Herbivory	1	5454	0.60	0.94
	CHG	Population	2	56698	3.81	**0.001**
		Herbivory	1	6882	0.93	0.60
	CHH	Population	2	44459	1.14	**0.001**
		Herbivory	1	22817	1.17	**0.001**
Artificial	CG	Population	2	96845	5.39	**0.001**
		Herbivory	1	5115	0.57	0.96
	CHG	Population	2	57282	3.83	**0.001**
		Herbivory	1	6317	0.84	0.78
	CHH	Population	2	45292	1.15	**0.001**
		Herbivory	1	20338	1.03	**0.05**

DIFFERENTIALLY METHYLATED CYTOSINES (DMCS) INDUCED BY HERBIVORY

The total numbers of DMCs, as well as the proportions of DMCs out of the total number of cytosines tested, found for each context, population and contrast (insect herbivory *vs.* control and artificial herbivory *vs.* control) are shown in Table 2. Overall, across all sequence contexts and populations, the total number of DMCs captured for insect and artificial herbivory was almost identical (11,339 and 11,319, respectively). When split by sequence context, Spanish samples had always a higher number of DMCs in response to insect herbivory in all contexts, whereas Italian and Polish samples showed higher numbers in response to artificial herbivory mainly in CG and CHG, but not in CHH. The number of DMCs captured in CHH context for both herbivory treatments was much lower than in the other two contexts. The proportion of hyper and hypomethylated DMCs was rather similar and close to 50% for CG and CHG in all three populations and both herbivory treatments (Table 2, Figure 3a). In the CHH context, however, DMCs from all populations were relatively more hypermethylated (methylation level increased) in response to the two herbivory treatments, except for the insect herbivory treatment from Spain that showed a greater proportion of hypomethylated DMCs (Figure 2a).

Table 2. Number of cytosines tested (Cs Tested), total number of differentially methylated cytosines (DMCs) detected (and percentage), and number of them that were hypermethylated and hypomethylated in samples experiencing insect or artificial herbivory compared to control samples in the three studied populations and for each sequence context.

Context	*Population*	*Herbivory*	*Cs Tested*	*DMCs*		
				Total (%)	*Hypermethylated*	*Hypomethylated*
CG	Spain	Insect	138,407	2,971 (2.2)	1442	1529
		Artificial	89,888	1,101 (1.2)	577	524
	Italy	Insect	171,002	1,072 (0.6)	580	492
		Artificial	175,989	1,681 (0.9)	739	942
	Poland	Insect	117,597	992 (0.8)	524	468
		Artificial	152,122	2,459 (1.6)	1459	2000
CHG	Spain	Insect	214,576	2,071 (0.9)	939	1132
		Artificial	138,552	718 (0.5)	391	327
	Italy	Insect	265,992	1,035 (0.4)	632	403
		Artificial	274,209	1,676 (0.6)	836	840
	Poland	Insect	182,779	724 (0.3)	408	316
		Artificial	235,699	1,684 (0.7)	972	712
CHH	Spain	Insect	1,138,341	637 (0.06)	243	394
		Artificial	724,963	352 (0.05)	218	134
	Italy	Insect	1,440,489	1,095 (0.08)	868	227
		Artificial	1,492,684	977 (0.07)	728	249
	Poland	Insect	956,131	742 (0.08)	610	132
		Artificial	1,253,171	671 (0.05)	444	227

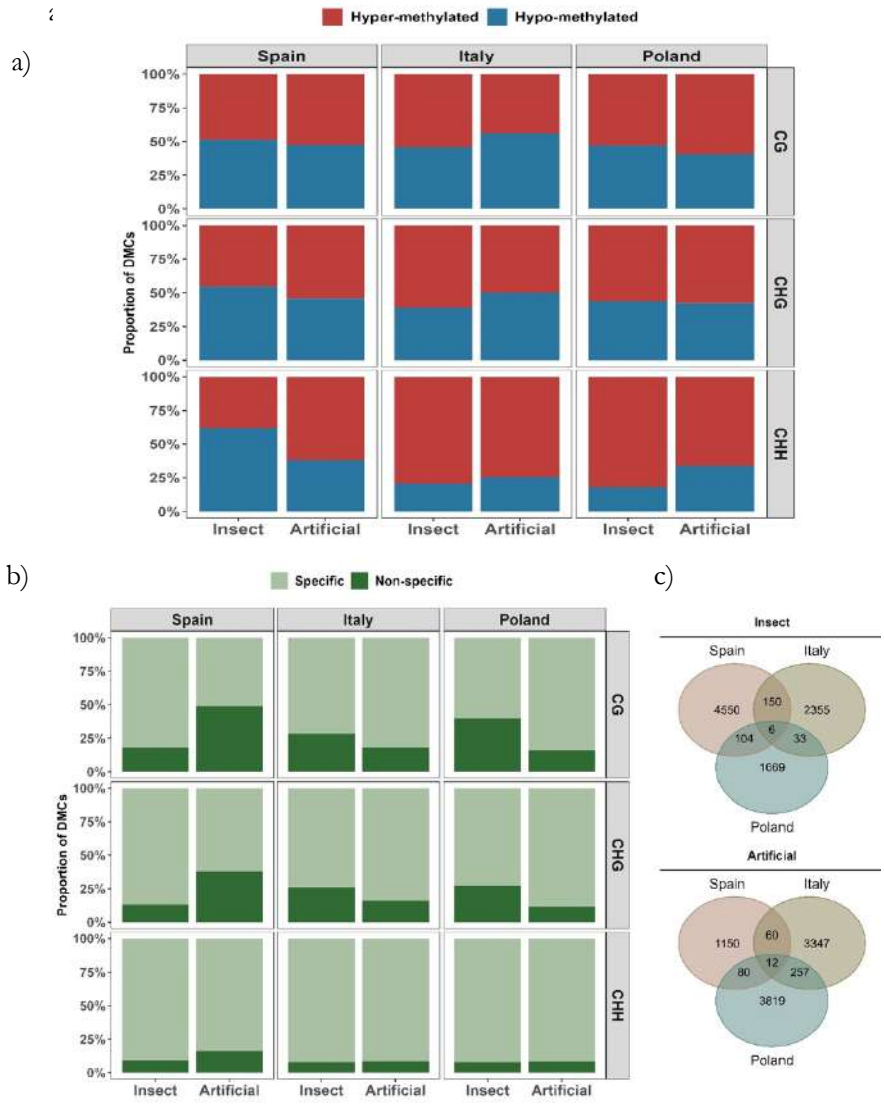

Figure 3. Differentially methylated cytosines (DMCs; minimum coverage of 10x, 10 % methylation change and q-value < 0.05) induced by insect and artificial herbivory in the three populations studied (Spain, Italy, and Poland) and the three sequence contexts (CG, CHG, and CHH). a) Proportion of DMCs (%) whose methylation level significantly increased (hypermethylated; red) or decreased

(hypomethylated; blue) in herbivory samples compared to control samples; b) Proportion of DMCs (%) that were specific to each herbivory treatment (stress-specific; light green) or common to the two herbivory treatments (non-specific; dark green); c) Venn diagram showing overlap of stress-specific DMCs between populations for insect and artificial herbivory (jointly for the three sequence contexts).

The vast majority of DMCs (between 60 and 92 %) captured in the three sequence contexts across all populations were stress-specific, that is, were induced either by insect or artificial herbivory (Figure 2b). This was especially true in the CHH context that showed the highest values of stress-specific DMCs in both contrasts (≥ 83 %; Figure 3b). In the CG context, populations showed slightly diverging trends, with ~ 50 % non-specific DMCs induced by artificial herbivory in Spanish samples and ~ 40 % in response to insect herbivory in Polish samples (Figure 3b). Interestingly, most of the stress-specific DMCs across the three sequence contexts were exclusively found in one population, i.e., were stress- and population-specific, and only ~ 0.1 % were shared by the three populations (6 and 12 respectively for insect and artificial herbivory; Figure 3c).

STRUCTURAL ANNOTATION OF DMCS INDUCED BY HERBIVORY

The relative distribution of the four genomic regions distinguished in the genome was estimated as ca. 31.15% for gene body, 19.6% for each of the two fixed flanking regions (promoter and downstream) and 29.65 for the intergenic regions (Dubay 2024). We found that the relative distribution of DMCs across them was rather similar across populations and contexts for the

two herbivory treatments (Table 3, Figure 4). In CG relative distributions of DMCs across the four genomic regions was overall similar to expected according to their relative abundance, DMCs induced by insect and artificial herbivory were predominantly located in gene body (> 27 %) and intergenic regions (> 30 %), whereas ≤ 19 % DMCs were mapped to either promoters or downstream regions. This pattern was rather consistent among the three populations studied, except for the fact that the proportion of DMCs in gene bodies seemed to be slightly overrepresented in ramets from Spain compared to the other two populations (Table 3, Figure 4a). In CHG, we found a lower representation in promoter and downstream regions (10-15 %) that was preferently shifted to gene bodies in Spanish ramets, intergenic regions in Italian ramets and equally to both features in Polish ramets. As regards the CHH context, a larger proportion of DMCs (≥ 48 %) were associated with intergenic regions for the two herbivory treatments, while DMCs mapped to gene bodies were variable among populations and overall less frequent (12-26 %) than in the other two sequence contexts (Table 3).

Table 3. Relative fraction (%) of DMCs associated to different genomic features (promoters, gene body, downstream, and intergenic regions), respect to total number of DMCs induced by insect and artificial herbivory in samples from Spain, Italy and Poland for the three sequence contexts.

Context	*Population*	*Herbivory*	*Genomic feature*			
			Promoter	*Gene body*	*Downstream*	*Intergenic*
CG	Spain	Insect	14.7	36.3	17.7	31.3
		Artificial	15.6	35.5	19.4	29.5
	Italy	Insect	15.7	27.6	18.6	38.1
		Artificial	17.0	28.2	19.0	35.8
	Poland	Insect	18.7	32.8	12.9	35.6
		Artificial	19.3	33.5	15.5	33.7
CHG	Spain	Insect	11.7	43.4	11.3	33.6
		Artificial	10.4	45.4	14.2	29.9
	Italy	Insect	12.6	33.2	13.5	40.7
		Artificial	12.8	36.3	11.2	39.7
	Poland	Insect	12.8	39.0	11.2	37.0
		Artificial	15.0	37.2	9.7	38.1
CHH	Spain	Insect	12.6	26.1	12.2	49.1
		Artificial	13.4	23.9	15.1	47.7
	Italy	Insect	12.2	12.0	9.9	65.9
		Artificial	11.9	14.3	11.0	62.8
	Poland	Insect	13.7	26.0	10.9	49.3
		Artificial	18.6	17.7	11.8	51.9

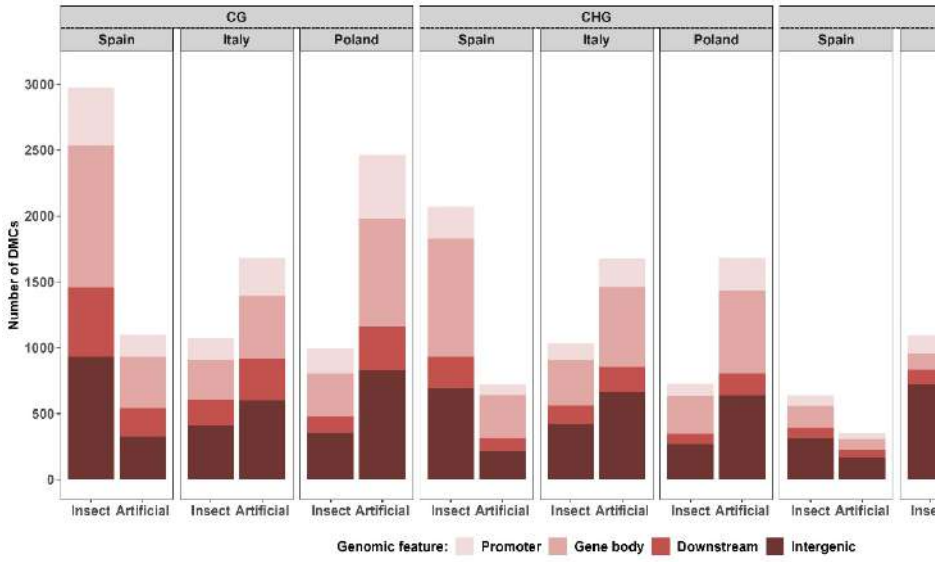

Figure 4. Structural annotation of differentially methylated cytosines (DMCs, N = 22,658) induced by insect and artificial herbivory according to the four genomic features distinguished (promoters, gene body, downstream, and intergenic regions) in samples from Spain, Italy and Poland for the three sequence contexts.

DMCS INDUCED BY HERBIVORY ASSOCIATED TO TRANSPOSABLE ELEMENTS

We also explored the association with transposable elements that collectively represent ca. 34.8% of the sequenced genome and found a total of 7,408 DMC-TEs (32.7% of the total number of DMCs found across populations and treatments), including 2,470 CG-, 2,570 CHG-, 2,368 CHH-DMCs (Table 4). As a general trend, in CG and CHG contexts DMCs were less frequently annotated as TEs (~21-37%) compared to CHH context (with ~ 43-60 % of DMC-TEs). On average, 58.9 % of the DMC-TEs were

hypermethylated and 42.1 % were hypomethylated. Interestingly, DMC-TEs in the CHH context tended to be slightly more hypermethylated regardless of the type of herbivory experienced, except for insect herbivory samples from Spain (Table 4).

Compared to the whole set of DMCs, a much smaller fraction of DMC-TEs were located into gene bodies (~ 14 %; Figure 5a). Moreover, in all populations, we found that most DMC-TEs overlapped (> 50 %) with DNA/Helitron and LTR/Gypsy TEs (Figure 5b). The LTR/Gypsy DMC-TEs were also highly represented in all three populations, especially in the CHG and CHH contexts. Specifically, DMCs in LTR/Gypsy TEs were found in higher ratio (~ 22 %) than reported in the *P. nigra* cv. italica reference genome (~ 15 %).

Table 4. Number of DMCs mapped to transposable elements (DMC-TEs) and their percentage respect to total number of DMCs induced by insect and artificial herbivory in samples from Spain, Italy and Poland for the three sequence contexts.

Context	*Population*	*Herbivory*	*DMC-TEs*			
			N	*%*	*Hypermethylated (%)*	*Hypomethylated (%)*
CG	Spain	Insect	627	21.1	305 (48.7)	322 (51.3)
		Artificial	235	21.3	119 (50.6)	116 (49.4)
	Italy	Insect	285	26.6	155 (54.4)	130 (45.6)
		Artificial	453	26.9	206 (45.5)	247 (54.5)

	Poland	Insect	261	26.3	118 (45.2)	143 (54.8)
		Artificial	609	24.8	326 (53.5)	283 (46.5)
CHG	Spain	Insect	591	28.5	298 (50.4)	293 (49.6)
		Artificial	202	28.1	106 (52.5)	96 (47.5)
	Italy	Insect	363	35.1	245 (67.5)	118 (32.5)
		Artificial	620	37.0	340 (54.8)	280 (45.2)
	Poland	Insect	250	34.5	134 (53.6)	116 (46.4)
		Artificial	544	32.3	271 (49.8)	273 (50.2)
CHH	Spain	Insect	333	52.3	144 (43.2)	189 (56.7)
		Artificial	150	42.6	95 (63.3)	55 (36.7)
	Italy	Insect	652	59.5	525 (80.5)	127 (19.5)
		Artificial	588	60.2	455 (77.4)	133 (22.6)
	Poland	Insect	321	43.3	248 (77.3)	73 (22.7)
		Artificial	324	48.3	202 (62.3)	122 (37.7)

a

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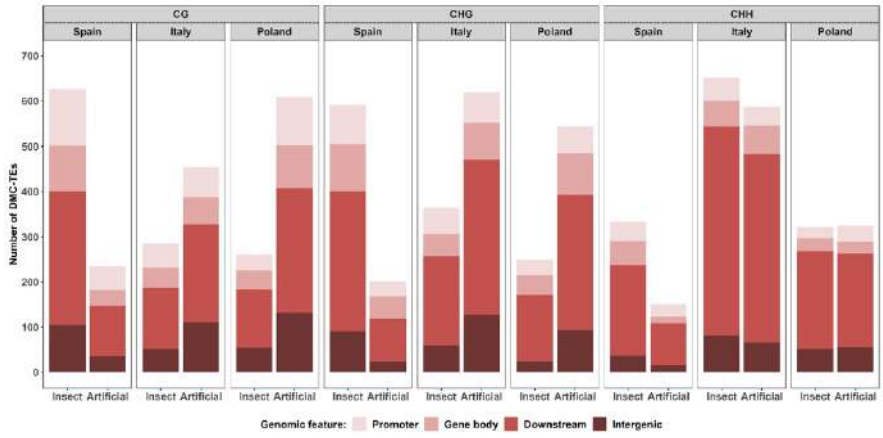

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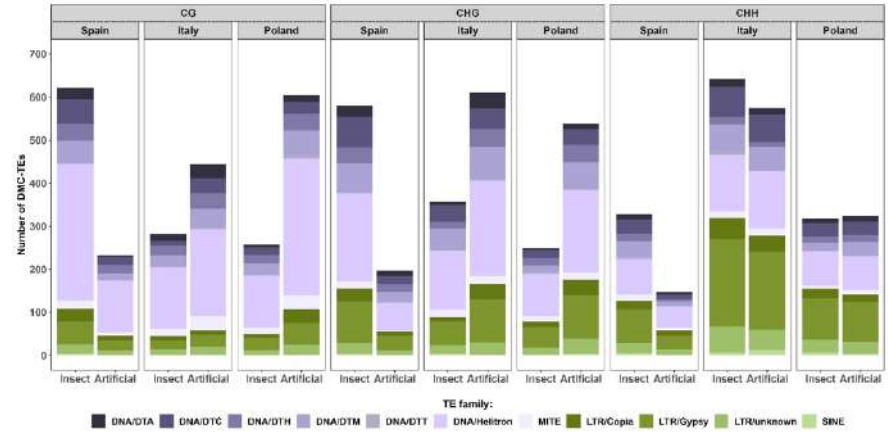

Figure 5. Characteristics of DMCs mapped to transposable elements (DMC-TEs, N = 7408) induced by insect and artificial herbivory in samples from Spain, Italy and Poland for the three sequence contexts. a) Structural annotation according to the four genomic features distinguished (promoters, gene body, downstream, and intergenic regions). b) Family identification of the DMC-TEs. Following the classification proposed by Peña-Pontón et al. (2024) based on TE predictions for *P. nigra* cv. *italica* reference genome, TEs were grouped into eleven of the most important superfamilies. In green class I TEs, or retrotransposons, and in purple class II elements, known as DNA transposons. Class I LTR: copia, gypsy, unk, SINE, Class II DNA: DTA, DTC, DTH, DTM, DTT, Helitron, Mite

"STRONGLY RESPONDING" DMCS

The distribution of "*strongly responding*" DMCs (i.e., top 5 % DMCs with the highest absolute methylation change across populations and treatments) along genic regions ranging from 4 kb upstream the transcription start site (TSS) to 4 kb downstream the transcription termination site (TTS) is shown in Figure 5. The abundance of these "*strongly responding*" DMCs across populations and treatments is quite uneven, with insect and artificial herbivory treated ramets from Spain, and artificial herbivory treated ramets from Poland accounting for most of them (Figures 6 and 7). The Italian population had the least number of DMCs with strong methylation differences between damaged ramets and controls, with almost 85% of DMCs having less than 20% methylation change (Figure 6). Moreover, "*strongly responding*" DMCs showed more hyper- than hypomethylation in artificial herbivory ramets from Poland, but tended to be more hypomethylated in artificial herbivory ramets from Spain (Figure 6). When split by context, in CG the number of "*strongly responding*" DMCs was slightly higher for insect herbivory ramets from Spain and for artificial herbivory ramets from Poland. Whereas, in CHH the strongest response was found in ramets from Spain after artificial herbivory (Figure 5). Across all populations and contexts, most of these "*strongly responding*" DMCs showed greater densities within gene bodies (i.e., they were located more frequently between TSS and TTS). Interestingly, all "*strongly responding*" DMC-TEs were located in gene bodies (Figure 6). Finally, across treatments, none of these "*strongly responding*" DMCs were shared among the three populations (Figure 7).

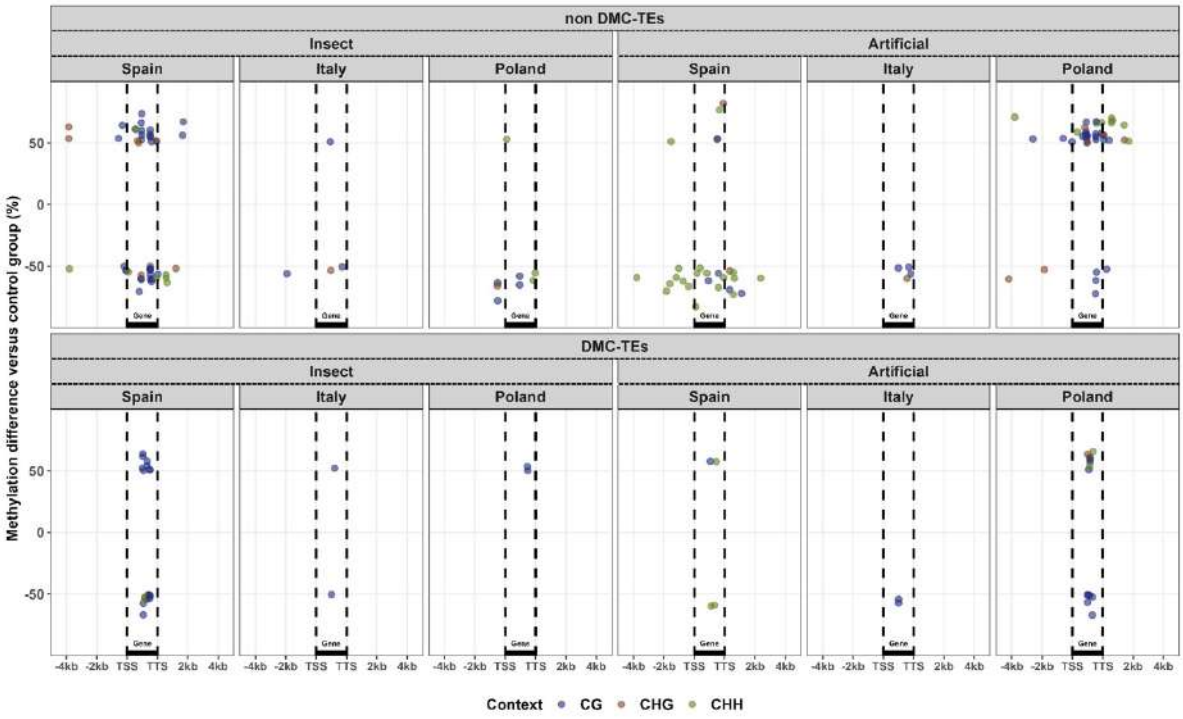

Figure 6. Detailed genomic distribution along genic regions of the "*strongly responding*" differentially methylated cytosines (top 5 % DMCs with the highest absolute methylation change across populations and treatments), per sequence context (CG: blue, CHG: brown, CHH: green), population (Spain, Italy, Poland) and herbivory treatment (insect, artificial). Upper panel shows DMCs not associated to transposable elements (non DMC-TEs). Lower panel shows DMCs associated to transposable elements (DMC-TEs). Vertical dashed lines delimit gene transcription start sites (TSS) and transcription termination site (TTS). The area between TSS and TTS represents the gene body. Gene lengths were normalized to 2 kb.

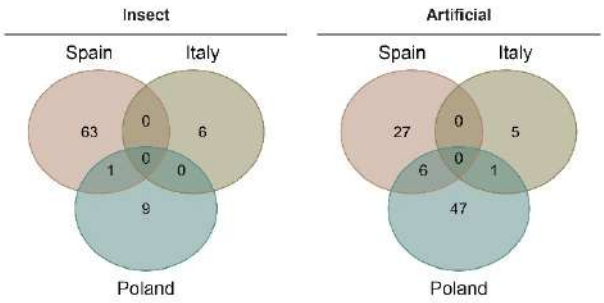

Figure 7. Venn diagram showing overlap of "strongly responding" DMCs (N = 173) between populations for insect and artificial herbivory (jointly for the three sequence contexts).

DISCUSSION

DNA methylation is a key player of many important biological cell processes, including genomic stability, euchromatin-heterochromatin distinction, gene expression regulation, and TEs silencing (Bucher et al., 2012; Law & Jacobsen, 2010; Zhang et al., 2018). It is also well-established that within plant species, individuals from distant geographic locations usually exhibit distinct DNA methylation profiles (see e.g., Bräutigam et al., 2013; Gugger et al., 2016; Galanti et al., 2022a). Particularly, in poplars, changes in DNA methylation occur during bud formation, dormancy and break associated to the seasonal leaf deciduous habit (Nunez-Martinez et al., 2024) and at a certain phenological stage, methylation profiles in leaf DNA can differ geographically (Díez-Rodríguez et al., 2022a), between nearby stands after controlling for genetic variation (see e.g., (Ahn et al., 2017) and even between branches of a single individual (Yao et al., 2021). Furthermore, individual exposure to stressful conditions caused by local climate change and/or antagonistic biotic interactions, can induce epigenetic changes in a short amount of time that can be relevant for both phenotypic adaptation and

genome evolution (Bucher et al., 2012; Zhang et al., 2018; Jueterbock et al., 2020; Lloyd & Lister, 2022). In particular, plant species with long generation times can undergo stress-induced changes in methylation patterns at specific genomic loci or across the entire genome, which aid in their defense mechanisms and adaptation potential (Thiebaut et al., 2019). Still, it is unclear how homogeneous is the epigenomic response to a certain environmental stress factor across individuals of a certain plant species (but see e.g., Chapter 2 this thesis; Herman & Sultan, 2016; Peña-Pontón et al., 2024). In this study, we investigate DNA methylation changes in response to experimental insect consumption and artificial leaf damage in young clones of *P. nigra* cv 'italica' propagated from trees found in three geographically distant European populations. Although we did not have previous information on defoliation records at the three tree provenances studied, we can expect some heterogeneity in past defoliation events between strands located in Spain, Italy and Poland (Charles et al., 2014). Thus, clonal propagation and a widespread distribution were ideal features to characterize genome-wide epigenetic variability in response to experimental stress in absence of genetic variation. In the following paragraphs, we will discuss our findings as regards common and distinct epigenetic alterations induced by either insect or artificial herbivory treatments across the three tree provenances and present the genomic location of those cytosines with extreme differential methylation between damaged and control ramets.

GENOME-WIDE DNA METHYLATION VARIATION IN GEOGRAPHICALLY DISTANT LOMBARDY POPLARS

In trees, both micro-environment and local herbivory can determine functional phenotypic variation in absence of genetic variation which is most likely associated to either transient or stable epigenetic variants (Díez

Rodríguez et al., 2022b; Herrera and Bazaga 2013). In poplar, DNA methylation was transmitted to the next clonal generation, but a fraction of the methylome changed relatively fast when comparing the parental individuals (i.e., ortets) with the clonal offspring (i.e., ramets) grown in a common environment (Díez Rodríguez et al. 2022b). A Europe-wide landscape analysis, including our three study populations, confirmed limited genetic diversity and absence of genetic population structure in the Lombardy poplar, whereas epigenetic divergence was larger and to some extent correlated with geographic distance (Díez Rodríguez et al., 2022a). Our RDA analysis showed a distinct population structure of DNA methylation in all sequence contexts, indicating a substantial epigenetic variation between the three European *P. nigra* populations here studied, and additional variance between ramets in methylation levels of loci in the CG, CHG, and CHH contexts. Such methylation variability might appear stochastically, by random epigenetic changes accumulated in plants at different origins (Díez Rodríguez et al., 2022b) and may contribute to increase phenotypic plasticity in asexuals under contrasting or stressful environments (Jueterbock et al., 2020; Rapp & Wendel, 2005; Verhoeven et al., 2010; Verhoeven & Preite 2014). In addition, exposures to similar stress or environmental conditions in the population of origin can result in epigenetic population structure that could influence how plant responds to a certain stress such as herbivory in subsequent seasons and generations (Bruce et al., 2007; Conrath et al., 2015; Dowen et al., 2012; Hilker & Schmülling, 2019). Altogether, in our study, we had reduced genetic variability and significant variation in DNA methylation across poplar clones obtained from Spain, Italy, and Poland.

INSECT AND ARTIFICIAL HERBIVORY INDUCED METHYLATION CHANGES SIMILAR IN SIGN BUT RECORDED AT DIFFERENT GENOMIC LOCATIONS

As regards the genome-wide effect of our experimental herbivory, we found that only methylation in CHH was responsive to herbivory treatments (see also Peña-Pontón et al., 2024; Xiao et al., 2021), and changes were more significant in response to insect herbivory than artificial mimicking. DNA methylation in CHH context is established *de novo* and it is typically transient (Boyko & Kovalchuk, 2010; Wibowo et al., 2016). Previous experimental studies also suggested that methylation in CHH is more plastic in response to several stress factors and in a variety of plant species including *P. nigra* (see e. g., Dubin et al., 2015; López et al., 2022; Peña-Ponton et al., 2024 and references therein). In our study, DNA in leaves of damaged ramets on average exhibited higher methylation levels than DNA of control ramets, although both increased and decreased methylation was frequently found at different loci (see below).

The epiGBS methodology allowed us to explore methylation changes at single nucleotide resolution and, thus, determine not only the sign of recorded changes but also the genomic location and potential association with genes or transposable elements that might help to link mechanistic understanding in the response to the experimental factors with the ecological and evolutionary consequences (Richards et al., 2017). This technique was particularly useful in a large experimental design as the one here presented because it produces similar output than whole-genome analysis, but then at only a small part of the genome and with an affordable cost (Chapter 4 this thesis; Gawehns et al., 2022; Troyee et al., 2023). Altogether, ca. 2 % of all cytosines tested were captured as DMCs, which roughly correspond to 300 – 3000 DMCs depending on the sequence context and paired comparison, that

are within the usual range of loci detected for experimental studies conducted with reduced representation analyses in other study systems (see e.g., Van Antro et al., 2023; van Gurp et al., 2016).

In any of the study populations, less than 30 % DMCs were shared among the two herbivory treatments suggesting that the effects of insect consumption were not perfectly mimicked by mechanical damage and JA-spraying (Hilker & Meiners, 2010) as previously observed at transcription level in black poplar (Babst et al., 2009; see also Major & Constabel, 2006). Furthermore, in the two symmetric contexts (CG, CHG) the Spanish samples had always a higher number of DMCs in response to insect herbivory, whereas Italian and Polish samples showed higher numbers of DMCs in response to artificial herbivory. This was, to some extent, unexpected, because the artificial herbivory treatment aimed to uncover the association between priming of defenses, frequently elicited by JA (see e.g., Zust & Agrawal, 2017), and changes in DNA methylation across study systems (see also Chapter 2 of this thesis). However, the response obtained in poplars was not homogenous across the three tree provenances, either in magnitude or in the specific loci whose methylation was altered (see next section).

GENOMIC LOCATIONS LINKED TO HERBIVORY-INDUCED METHYLATION

Compared to other organisms, plants show broader variance in global DNA methylation levels, with non-CG methylation being frequent and predominantly found out of genes (Kenchanmane Raju et al., 2019). Thus, not surprisingly, functional consequences of methylation changes have been associated to genomic location, sequence context and the sign of change (Lloyd & Lister, 2022). According to previous studies, mainly in *Arabidopsis thaliana*, TEs are highly methylated in all sequence contexts when silent and,

thus, a reduction in methylation may activate TEs. However, reduced methylation in genes may increase transcription or the opposite effect depending on the sequence context and whether it is nearby the starting transcription site (CG) or at gene body for CG and CHG context (Lloyd & Lister, 2022). In our study, DMCs induced by insect and artificial herbivory in CG and CHG were predominantly located in gene bodies and their 2 kb flanking regions, and increased and decreased methylation was similarly frequent. Further, DMCs in CHH were predominantly found at intergenic regions, frequently associated to TEs and overall tended to exhibit increased methylation in damaged plants, again supporting that methylation at CHH was the most responsive in our study system (see also Peña-Ponton et al., 2024) for the effect of drought). Congruently, differential non-CG methylation was found to be more strongly associated with TEs in response to leaf damage in *Mimulus* (Colicchio et al., 2018).

Altogether, our differential methylation analyses suggested that epigenetic regulation after herbivory may involve changes not only in gene expression but in TEs activity as well (Mirouze & Paszkowski, 2011), as expected for the relevance of TEs in plant genomes (Springer et al., 2016). In *Populus* species, TEs make up 35–40% of the genome with class I or RNA (retroelements) making up 10% and class II or DNA transposon making ca. 2-3 % (Ramakrishnan et al., 2021b). Our study showed that a large proportion of DMCs overlapping with TEs were associated to the LTR-Gypsy and Helitron families, mostly in the CHH context This is partly due to the abundance of these TE superfamilies in the *P. nigra* genome, although provisional, hypergeometric tests with our data pointed to significant enrichments for the LTR-Gypsy, LTR-Copia, and Helitron superfamilies (results not shown). The LTR-Gypsy are retrotransposons (class I) that

mobilize through an RNA intermediate via a "copy-and-paste" mechanism (Bucher et al., 2012), and they appear to be activated by stressful conditions such as high salinity and heat in other plant species (Liu et al., 2021; Miryeganeh et al., 2022; Wang et al., 2018). The Helitron mobilize by a "cut-and-paste" method (class II) and are very changeable under stress, directly affecting neighboring gene transcription (stress-responsive genes) when inserted (Zhao et al., 2022).

If we pay attention to "*strongly responding*" loci, no overlapping existed in DMCs obtained in samples from different provenances, and very few were obtained in ramets from Italy. In CG, we found a similar tendency to increased and reduced methylation mainly associated to gene bodies either in response to insect (Spain) or artificial herbivory (Poland). In CHH, decreased methylation was frequently observed in Spanish ramets after artificial herbivory both in gene bodies and in their flanking regions. While the functional relevance of gbM in response to stress remains unclear, it has been associated with gene expression flexibility in some studies (Bewick & Schmitz, 2017b; Wang et al., 2021) and, other studies suggest that gbM may be a passive byproduct of other epigenetic processes within genes (Wendte et al., 2019). Here, we showed that all "*strongly responding*" DMCs associated to TEs were located within gene bodies suggesting that changes elicited by herbivory might be related to de/activation of TEs inserted within genes. Thus, an integrative analysis of transcriptome and small RNAs might be required to better interpret our results and improve current understanding of the epigenetic mechanisms behind heterogeneous systemic induced defenses both within and across different plant species (see e.g., Babst et al., 2009; Colicchio et al., 2018; Colicchio & Herman, 2020; Dugé de Bernonville et al., 2020)

CONCLUSION

To sum up, our experimental study in Lombardy poplar showed that (i) in the control samples, a significant amount (17.2%) of variation in DNA methylation at all three contexts (CG, CHG, CHH) was explained by the population of origin; (ii) a significant increase on DNA methylation of undamaged and completely expanded leaves produced after the first priming event appeared only in CHH contexts, when the effects of insect and artificial herbivory were analyzed independently across populations. Furthermore, the combination of those two elements suggests that the response to the specific herbivory experienced may vary with plant origin because (iii) in response to insect herbivory, ramets from Spain responded more than those from the other two populations, whereas for the artificial herbivory, ramets from Poland were the most responsive, and Italian plants exhibited the least response in the two cases. Finally, we explored the genomic location of main changes and found that (iv) DMCs were predominantly recorded in gene bodies and their flanking regions (CG, CHG) or associated to intergenic regions (CHH). Around one third of DMCs were in TEs, with > 50 % of these overlapping with DNA/Helitron and LTR/Gypsy TEs for both herbivory types. Overall, these results suggest that systemic response to insect herbivory involves changes in DNA methylation that are widespread along the genome, relatively specific and geographically heterogeneous.

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CHAPTER 4: HERBIVORY INDUCED METHYLATION CHANGES IN THE LOMBARDY POPLAR: A COMPARISON OF RESULTS OBTAINED BY EPIGBS AND WGBS

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ABSTRACT

DNA cytosine methylation is an epigenetic mechanism involved in regulation of plant responses to biotic and abiotic stress and its ability to change can vary with the sequence context in which a cytosine appears (CpG, CHG, CHH, where H = Adenine, Thymine, Cytosine). Quantification of DNA methylation in model plant species is frequently addressed by Whole Genome Bisulfite Sequencing (WGBS), which requires a good-quality reference genome. Reduced Representation Bisulfite Sequencing (RRBS) is a cost-effective potential alternative for ecological research with limited genomic resources and large experimental designs. In this study, we provide for the first time a comprehensive comparison between the outputs of RRBS and WGBS to characterize DNA methylation changes in response to a given environmental factor. In particular, we used epiGBS (recently optimized RRBS) and WGBS to assess global and sequence-specific differential methylation after insect and artificial herbivory in clones of *Populus nigra* cv. 'italica'. We found that, after any of the two herbivory treatments, global methylation percentage increased in CHH, and the shift was detected as statistically significant only by epiGBS. As regards to context-specific differential methylation induced by herbivory (cytosines in epiGBS and regions in WGBS), both techniques indicated the specificity of the response elicited by insect and artificial herbivory, together with higher frequency of hypo-methylation in CpG and hyper-methylation in CHH. Methylation changes were mainly detected in gene bodies and intergenic regions in CpG and CHG and in transposable elements and intergenic regions for CHH. Thus, epiGBS succeeded to characterize global, genome-wide methylation changes in response to herbivory in the Lombardy poplar. Our results support that epiGBS could be particularly useful in large experimental designs

aimed to explore epigenetic changes of non-model plant species in response to multiple environmental factors.

INTRODUCTION

DNA methylation is an epigenetic modification found in the genomes of most living organisms, from bacteria to plants, animals and fungi (S. Feng et al., 2010; Law & Jacobsen, 2010). Cytosine methylation is the most common form of DNA methylation and it indicates the addition of a methyl group (CH3) at 5'-carbon pyrimidine ring of cytosine nucleotide (Law & Jacobsen, 2010), hereafter referred to as DNA methylation. The frequency and location of DNA methylation vary drastically among animals and plants. In animals, it predominantly occurs on guanine- and cytosine-rich regions of the genome, specifically in CpG context, while in plants, DNA methylation is more extensive and can also be found in CHG and CHH (H = Adenine, Thymine, Cytosine) contexts, catalyzed by a diverse set of methyltransferase enzymes (Law & Jacobsen, 2010; Niederhuth et al., 2016). Plants exhibit widespread variation in DNA methylation among and within plant families. Specifically, the global DNA methylation level in angiosperms has been linked to variance in genome size (Alonso et al., 2015; Niederhuth et al., 2016) and to some extent to other intrinsic and extrinsic factors such as life history traits and regional provenance (Alonso et al., 2019; Verhoeven & Preite, 2014). Regarding the relative frequency of DNA methylation in different sequence contexts, methylation is ubiquitous and relatively frequent in CpG context for the majority of plant species, whereas methylation levels in CHG are reduced in species of the Brassicaceae family, and tend to be low for CHH in the Poaceae family (Niederhuth et al., 2016). As regards the location within

genomes, methylation in the three contexts is highly enriched in repetitive DNA, transposons, and pericentromeric regions, whereas genic methylation tends to occur mainly in the CpG context (Muyle et al., 2022; H. Zhang et al., 2018).

At the intraspecific level, variation in DNA methylation has been linked to geographic and environmental gradients in wild plant populations (see e.g., (Alonso et al., 2016; De Kort et al., 2021; Galanti et al., 2022; Kawakatsu et al., 2016)). Furthermore, sound evidence for changes in DNA methylation associated to abiotic stress (Alonso et al., 2016; Herman & Sultan, 2016) and biotic interactions have been predominantly recorded in model or crop plants (reviewed in (Ramos-Cruz et al., 2021)). In particular, DNA methylation variation has been associated with plant defense against herbivores in several species (Herrera & Bazaga, 2011; Rasmann et al., 2012; Rendina González et al., 2018; Sobral et al., 2021; Troyee et al., 2022). For example, in *Brassica rapa*, foliar herbivory is associated with both DNA demethylation and changes in pollinator-relevant floral traits that decrease the attractiveness of the plants to their main pollinators (Kellenberger et al., 2016). Most of the referred studies above have used either global estimates of methylation or anonymous markers to detect overall changes in species lacking a reference genome, impeding the ability to infer the genomic location and potential function of observed methylation changes in response to insect or artificial herbivory (but see for instance (Colicchio et al., 2018; Scoville et al., 2011).

Whole Genome Bisulfite Sequencing (WGBS) and Reduced Representation Bisulfite Sequencing (RRBS) are two methods for studying DNA methylation, with RRBS being a cost-effective method for quantifying methylation that targets a small and restriction enzyme site-associated fraction

of the genome (Paun et al., 2019). Bisulfite sequencing (BS-seq) includes pre-treating DNA with sodium bisulfite, which converts cytosine to uracil while 5-methylcytosine remains unchanged, followed by sequencing and data processing to identify methylation at single cytosine site resolution (Gu et al., 2011; Lister & Ecker, 2009). WGBS can cover entire genome and is regarded as the gold standard for studying DNA methylation because its single-base resolution provides information about the sequence context and offers the possibility for mapping to entire genomes. The quality of the output will vary with genome features (e.g., genome size, frequency of repeats), sequencing depth reached and the quality of the annotated reference genome, which limits its application to non-model plants with unknown genome features (Paun et al., 2019). RRBS, in contrast, targets a reduced fraction of the genome using restricted digestion that allows studying species with varying genome sizes and species that lack a reference genome at a lower cost. Among the various RRBS techniques available, epiGBS (Paun et al., 2019; van Gurp et al., 2016) is a method with specific barcoded adapters that uses genotyping by sequencing of bisulfite-converted DNA . Its bioinformatics analyses provides a reliable de novo reference for the genomic loci that are targeted by the method, or use an existing reference genome, for inferring both methylation quantification and single nucleotide polymorphism detection (Gawehns, Postuma, Antro, et al., 2022; van Gurp et al., 2016). EpiGBS has been successfully used to estimate overall DNA methylation variation in a variety of non-model species, including mosses, snails, birds, and plants (Boquete et al., 2021; Luviano et al., 2021; Mcnew et al., 2021; Mounger et al., 2021; van Gurp, 2017). Furthermore, estimation of DNA methylation by epiGBS correlates well with estimates obtained by WGBS for several accessions of the model species *Arabidopsis thaliana* (Gawehns, Postuma, Antro, et al., 2022). Nonetheless, since epiGBS inherently captures only a

certain fraction of the genome, it is unknown how effective the method is at capturing the methylation response to specific stress factors (Paun et al., 2019). Therefore, to move forward in analyzing the epigenetic contribution to plant responses to stress, it is crucial to corroborate how similar are the outputs of epiGBS and WGBS to comprehend specific stress responses in non-model plants. If the two techniques identify similar global and context-specific methylation shifts and point to similar genomic location of most of the observed methylation changes in response to a certain level of environmental stress, the epiGBS analyses could be useful to explore the links between epigenetic variation and plant functional phenotypic traits, with typical ecological designs involving large sample sizes and multiple levels of variation that have mainly used anonymous markers (Herrera & Bazaga, 2011; Rendina González et al., 2018; Troyee et al., 2022) or indirect evidence of epigenetic contribution to stress response (Herman & Sultan, 2016).

In this study, we used both WGBS and epiGBS techniques to evaluate changes in DNA methylation in cuttings of the Lombardy poplar, *Populus nigra* cv. 'italica', in response to insect herbivory and artificial herbivory. Lombardy poplars are fast-growing trees that have a clonal origin and widespread distribution, which makes this cultivated variety an excellent study system to investigate epigenetic responses to specific factors with reduced variation at the genetic level. Furthermore, in long-lived plants, rapid and reversible methylation changes can contribute to plant phenotypic plasticity (Bräutigam et al., 2013; Carbó et al., 2019; Sow et al., 2021) and could perhaps be associated with transcription changes observed after insect and artificial herbivory in poplar (Babst et al., 2009). Specifically, here we addressed the following questions: i. To what extent genome-wide methylation changes induced by herbivory can be similarly detected using epiGBS and WGBS

outputs? ii. How frequent and strong are these methylation changes in the three sequence contexts according to the two techniques? iii. Are there technical/biological biases between the two techniques with regard to the frequency of herbivory-induced methylation variations in specific genomic sites (gene body, gene promoter, transposable element, etc.)? iv. Can the two techniques identify functional changes associated with the plant methylation response to herbivory?

METHODS

STUDY SYSTEM

Plant species

Poplar is a suitable genus for genome-wide investigations due to its compact genome size (~500 Mb) and availability of reference genomes (Pinosio et al., 2016). Black poplar, *Populus nigra* L. (Salicaceae), is a diploid deciduous tree native to north-west Europe that grows in floodplain woods and riparian environments (Dickmann & Kuzovkina, 2014). We used the clonal Lombardy cultivar (*Populus nigra* cv. 'italica' Duroi) because it can be easily propagated by cuttings and has low genetic variation (Rodríguez et al., 2022). We did not require any special permit for sample collection because our study plants came from the Marburg Botanical Garden (Germany).

Herbivory types

We used two forms of tissue damage: true insect herbivory and simulated artificial herbivory to decipher the changes induced by herbivory in terms of DNA methylation. Larvae of the gypsy moth, *Lymantria dispar* (L.) (Erebidae), were used for insect herbivory treatment. This polyphagous insect

is a major pest of northern hemisphere hardwood forests, fruits, and ornamentals, that has over 500 host plant species including poplars (Mccormick et al., 2014), it has a short generation time with precisely defined developmental stages and larvae that are easy to manipulate. We obtained L2 instar larvae from Dr. Sybille Unsicker's lab (Max Planck Institute for Chemical Ecology, Jena, Germany) and kept them in a climate chamber (14/10 h light/dark, 20 C, 60% humidity) feeding on artificial diet (MP Biomedicals LLC) until the experiment was conducted. Two-three days before the start of the herbivory treatment, larvae were fed poplar leaves to get them adapted to this food source. For the insect treatment, larvae were always placed on fully expanded leaves (see below for further details). The artificial herbivory treatment was performed by mechanically punching holes in the leaves and spraying them with a solution of Jasmonic Acid (JA), which is known to be an important chemical elicitor of many plant defense responses (Thaler et al., 1996). These two complementary treatments were selected because *Populus* species develop secondary defense chemicals in response to jasmonates (Babst et al., 2009). Also, when black poplar is attacked by gypsy moth caterpillars, it produces a variety of direct and indirect defenses, including an increase in JA on damaged leaves and several volatile organic compounds that attract herbivore enemies (Fabisch et al., 2019; Mccormick et al., 2014).

EXPERIMENTAL DESIGN

Plant Materials and Growth Conditions

Lombardy poplar clones were generated through vegetative propagation by rooted cuttings from three adult parent trees located in Italy and grown in a common garden in the Marburg Botanical Garden (Germany)

for 10 months (Rodríguez et al., 2022). Each garden planted tree from which all the members of a clone have descended will be denoted hereafter as an ortet. Cuttings of approximately 30 cm in length were sampled from the common garden trees and stored at 4 ºC and dark conditions for 2 weeks. Cuttings were soaked (2 cm bottom) in a rooting solution (50 mg/L Rhizopon AA 50 mg tablets) overnight and planted in 2L pots (3 cuttings per pot) with 1:1 sand:peat mixture (30% coarse sand, 20% fine sand, and 50% nutrient-poor potting soil) and 5 ml of rooting solution. Pots were maintained in a flood table with regular watering to pot capacity for 2 weeks and then rooted cuttings were transplanted to individual 2 L pots with the same 1:1 sand:peat mixture and watering regime. Cuttings were fertilized with slow-release fertilizer Osmocote Exact mini (15-9-11+2MgO+TE) after two weeks (2 g) and 10 weeks (1 g) of transplanting. Greenhouse conditions during the experiment included: temperature: (day/night) 22/18 °C (±2°C), relative humidity: 60% (±5%), light: (day/night) 16/8 h.

A total of 27 similar-sized and 15-week-old cuttings (ramets hereafter) originated from the three ortets (i.e., nine ramets per ortet) were used in the experiment. Ramet is defined as an individual obtained clonally from an ortet, and thus, the nine ramets derived from a certain ortet should be genetically identical individuals. Three ramets per ortet were included in each of the three experimental treatments: control, insect herbivory, and artificial herbivory. We did not assort the plants from different treatments together in order to avoid volatile organic compounds exchange (Mccormick et al., 2014). Per treatment, the ramets were randomly distributed in two trays (flood tables) and were randomized regularly.

Herbivory Treatments Procedure

To control for potential positional effects, damage was always inflicted on leaves of the basal half of the main branch of each ramet and methylation changes will be determined from material taken from the most adjacent undamaged leaves grown in the apical half of that branch. Both insect and artificial herbivory treatments were repeated twice to enable a priming effect to finally get a stronger and/or faster response (Mauch-Mani et al., 2017; Rasmann et al., 2012). For priming the plants in insect herbivory treatment, ten *L. dispar* L2 instar caterpillar's larvae were placed on full expanded leaves of the main branch of our experimental poplar ramets, which were enclosed using nylon mesh bags (75*100 cm). After five days, the larvae were removed for three days so the plants could recover. For second herbivory induction event, seven L2 instar and five L4 instar larvae were placed on leaves of the lower part of the main branch, enclosed within a nylon mesh bag (75*100 cm) and allowed to feed freely for seven days before the collection of leaf samples (see below).

Artificial herbivory was conducted in the main branch and in a similar location as insect herbivory. In the priming phase, 6-8 holes per leaf (ca. 3-5 mm diameter) were punched in three to four leaves of the basal half of the main branch. Immediately after the artificial wounding of the leaves, two pumps (150 μL/pump) of a JA solution were sprayed on the damaged leaf and three pumps all over the plant, repeating this procedure two times. During the second herbivory event similar number of holes as for priming were punched in each of 10-12 leaves and JA solution was sprayed four-times and enclosed within nylon mesh bags as described above. The JA was solubilized in ethanol and diluted in deionized water to a 1-mM JA (Sigma J2500- 100MG) solution with 0.1% Triton-x 100 as a surfactant to increase cuticle penetration (Babst et al., 2009).

In the control group, similar-positioned, well-developed leaves from the main branch of each ramet were sprayed with an equivalent aqueous solution in which no JA was added and covered with nylon mesh bags in a similar manner as the herbivore treated ones. The experiment finished 17 weeks after clonal propagation.

LABORATORY METHODS AND LIBRARY PREPARATION

Sampling and DNA Extraction

We collected tissue from undamaged and fully expanded leaves of the adjacent apical half of each ramet, either 24 hours after the second herbivory event in treated plants or after the aqueous spraying in controls. We kept these leaves without any bag cover throughout the duration of the experiment. A cork borer was used to take 5-6 discs of leaf tissue (ca. 3-5 mm diameter), that were stored in labelled vials and immediately frozen in liquid nitrogen. Vials were kept at -80 °C until DNA extraction. Sampling and DNA extraction order were determined by randomly selecting one sample per treatment (irrespective of the ortet) at a time. Frozen leaf material was disrupted and homogenized using a Qiagen TissueLyser II with two stainless steel beads (45 seconds at a frequency of 30.00 1/s). Macherey-Nagel NucleoSpin Plant II kit was used to do DNA extraction and cell lysis Buffer PL1 (CTAB method) was used to get optimum DNA quality. For each sample, an aliquot of 35 µl at 30 ng/µl was used for epiGBS and at least 1 µg of DNA from a stock solution at minimum 20 ng/µl was used for WGBS. It is important to remark that this is the first time that plant DNA from identical plant individuals has been analyzed using these two techniques.

epiGBS Library Construction, Sequencing and Pre-Processing

We followed the epiGBS2 protocol with few modifications (Gawehns, Postuma, van Antro, et al., 2022). Samples were randomized and DNA digested using restriction enzymes AseI and NsiI. Hemi-methylated adapter pairs containing barcodes of sample-specific 4-6 nucleotides were then ligated to the digested DNA and the barcodes are usually followed by three random nucleotides. Additionally, an unmethylated cytosine is added at the end to annotate Watson and Crick strands to estimate the bisulfite conversion rate. Next, the samples were multiplexed, concentrated and smaller fragments (<60 bp) were removed by NucleoSpin Gel & PCR cleanup Kit. SPRIselect magnetic beads were used to select DNA fragments of 300 bp (and lower). Deoxynucleoside triphosphates (dNTPs) that contain 5-methylcytosine were used to repair the nicks produced by hemi-methylated adapters to obtain completely ligated and methylated adapters. We used the EZ DNA Methylation-Lightening kits protocol for converting the multiplexed samples. PCR-amplification of converted DNA was done followed by a final clean-up and size selection. The obtained library was then sequenced paired-end (PE 2x150bp) in one lane of an Illumina HiSeq 4000 sequencer with a 12% phiX spike.

Both the 'reference' branch and '*de novo*' branch epiGBS2 pipeline were used to analyze sequencing data (Gawehns, Postuma, Antro, et al., 2022). All the steps were embedded in a Snakemake (version 6.1.1) workflow (Köster & Rahmann, 2012). Firstly, removal of PCR duplicates was performed based on the inserted 3-random nucleotide sequence in the adapter sequences. This step was done to confirm true PCR clones so it can be removed from the sequencing data but not the biological duplicates. Then Stacks 2 software (Rochette et al., 2019) was used to demultiplex the samples

in accordance with the barcodes followed by adapter trimming using Trim Galore! (version 0.5.0) and cutadapt (data stored in European Nucleotide Archive (ENA) project: PRJEB51853). The bisulfite conversion rate, estimated based on the number of correctly bisulfite-converted control cytosines within the adapters (see Gawehens et al., 2022 and above), was found globally satisfactory (≥ 94.88 %). The pipeline then maps the sequence fragments of experimental data with the reference or consensus genome using Bismark v0.19.0 (Krueger & Andrews, 2011) with the default settings. In the epiGBS2 reference branch (epiGBS-R hereafter), the *P. nigra* cv 'italica' reference genome (available at ENA project: PRJEB44889) was used for mapping with default parameters (sequence identity in the last clustering step). In epiGBS *de novo* branch (epiGBS-D hereafter) the genome generated from consensus clusters was used to map the fragments. The final output was a Bismark report file for each sample that contains lines with chromosome/scaffold name, genomic position, strand information, methylated cytosine number, unmethylated cytosine number, cytosine sequence context name (CG/CHG/CHH) and trinucleotide context information (where H are indicated by true Adenine, Thymine, and Cytosine in the sequence). For the epiGBS library, 240 million (243,507,236) reads were successfully demultiplexed and assigned to individual samples (N = 27 ramets). In epiGBS-D, a de novo assembly produced 106,267 clusters of 32-290 bp in length (mean = 224 bp), with an average of 1.4 fragments per contiguous cluster (minimum = 1 and maximum = 437).

WGBS Library Construction, Sequencing and Pre-Processing

Preparation of DNA libraries for bisulfite sequencing was performed by IGA Technology Services (Italy) using the Ovation Ultralow Methyl-Seq System (NuGEN, Redwood City, CA) following the manufacturer's

instructions. Library preparation order followed the same randomized sample design as for DNA extraction. Libraries were sequenced paired-end (PE 2x150bp) at approx. 25X coverage on an Illumina NovaSeq6000 sequencing system. Libraries were randomized and sequenced in two lanes. The sodium bisulfite non-conversion rate was calculated as the percentage of cytosines sequenced at cytosine reference positions in the chloroplast genome and it was found globally satisfactory with conversion 96.37%.

Sequenced reads were processed using the EpiDiverse Toolkit (WGBS pipeline v1.0, https://github.com/EpiDiverse/wgbs) (Nunn et al., 2021). Briefly, low-quality read-ends were trimmed (minimum base quality: 20), sequencing adapters removed (minimum overlap: 5 bp), and short reads (<36 bp) discarded. The remaining high-quality reads were aligned to the *P. nigra* cv. 'italica' reference genome mentioned above using erne-bs5 alignment package (http://erne.sourceforge.net) allowing for 600-bp maximum insert size, 0.05 mismatches, and unique mapping. Per-cytosine methylation metrics were extracted using MethylDackel (https://github.com/dpryan79/MethylDackel). Three bedGraph files per sample were produced, corresponding to cytosines on each sequence context: CpG, CHG and CHH. Each of these files contained a matrix where each line consists of six columns that indicated the scaffold name, start coordinate, end coordinate, methylation percentage, number of alignments reported methylated bases and unmethylated bases. For the WGBS library, after adaptor trimming and quality control 200 million (200,660,447) reads per sample were processed, and a total of 52 million (52,885,999) reads were demultiplexed, assigned to individual samples and mapped for further analysis.

epiGBS and WGBS Data Filtering

The downstream analyses required to estimate changes in methylation status between study groups were conducted with methylKit (Akalin et al., 2012) based on Bismark report files. Bismark file of every sample was checked for global low read coverage based on the retained positions and approved since more than 50% of total positions had sufficient read depth in all cases. Next, cytosine loci with five or fewer sequencing read depths for WGBS (6x) and nine or fewer reads for epiGBS-D and epiGBS-R (10x) were removed and stored as flat file databases. We applied different minimum read coverage because data from the two sequenced libraries indicated that epiGBS got higher coverage than WGBS for the captured cytosines (S1 Fig). Individual databases were later merged using the *unite* function of methylKit that kept bases covered by 2/3 of the samples per treatment group (i.e., in six out of nine samples). We retained a total of 1,823,024 (epiGBS-R), 1,148,755 (epiGBS-D) and 116,785,713 (WGBS). Furthermore, in order to compare only positions with methylation calls that were common to all samples, a dataset was built without any missing values (i.e., data available in 100% of samples). The number of cytosine positions in the three sequence contexts captured by each technique after read coverage filtering and the two sample representation options are shown in Table 1.

Table 1. Total number of cytosines captured by epiGBS-R , epiGBS-D and WGBS techniques in the three sequence contexts (CpG, CHG and CHH) according to the sample representation threshold applied.

		Sample representation		
		2/3 samples	all samples	
Technique	**Sequence context**	**No. cytosines***	**No. cytosines***	**%**
epiGBS-R	**CpG**	167,193	24,126	14.43
	CHG	260,884	36,033	13.81
	CHH	1,394,947	172,631	12.37
epiGBS-D	**CpG**	108,462	62,456	57.58
	CHG	162,351	92,952	57.25
	CHH	877,942	457,165	52.07
WGBS	**CpG**	10,530,726	210,014	1.99
	CHG	16,019,281	330,541	2.06
	CHH	90,235,706	963,809	1.07

* Values indicate the total number of cytosines included in each dataset after doing the minimum read coverage filtering (≥5 sequencing coverage in WGBS; ≥10 in epiGBS-R and epiGBS-D), taking into account their presence in at least 2/3 of study samples per group or being common to all study samples.

DATA ANALYSES

Methylation Levels in epiGBS and WGBS

Estimates of methylation levels in the three sequence contexts (CpG, CHG, CHH) and genomic features (promoters, gene bodies, intergenic region and corresponding transposable elements) were calculated for positions that were common to all samples using epiGBS-R, epiGBS-D and WGBS data. Methylation level (%) of a particular site was calculated as: (methylated cytosine read count)/(methylated cytosine read count + unmethylated cytosine read count) * 100. The average methylation was subsequently estimated as the mean of all sequenced cytosines in a sample. Average methylation percentage in each context was compared among epiGBS-D, epiGBS-R and WGBS by Pearson's Chi-square test using *chisq.test* function of stats R package v3.6.2). Divergence in average methylation (%) between study samples was evaluated independently for each technique with a linear model including herbivory (with three levels) and ortet (with three levels) as fixed factors. Significance of fixed factors and their interaction was tested using the function ANOVA (package car, v3.0.12) (J. Fox & S. Weisberg, 2019). Additionally, we have conducted a technical analysis with the common fragments obtained by both epiGBS-R and WGBS, i.e. using only the subgroup of fragments that had identical coordinates when mapped to the reference genome (average fragment size of 204 bp). But since the results of this technical comparison are very specific and does not change substantially our main conclusions, to improve readability and conciseness, they are provided as a supplementary material (S1 Appendix).

DIFFERENTIALLY METHYLATED CYTOSINES (DMCS)

In addition to individual loci filtering, we removed all positions where the sum of methylated reads across all samples was less than 10 (i.e., very low methylated positions) from the merged file (see epiGBS and WGBS data filtering section in Methods). Monomorphic positions in our dataset, i.e. those that were always unmethylated or fully methylated were removed. The filtered dataset was analyzed with generalized linear models as implemented in the R package methylKit that assumes that the methylated to unmethylated counts follow a binomial distribution and the effect of the fixed factors can be estimated with a log-likelihood test for logistic regression (Akalin et al., 2012). MethylKit allows parameter adjustment to identify DMCs corrected by multiple testing based on *q*-value (*q*-value <0.05), the minimum methylation difference (fixed = 10 %), and direction of methylation shift (hyper or hypo). DMCs were called separately for insect and artificial herbivory treated plants in comparison with control plants, each model included herbivory (control *vs* treated) as fixed factor and ortet as covariate. Finally, we searched for "stress-specific" DMCs that were present only in one type of herbivory and "non-specific" DMCs that were common to both herbivory treatments. The same criteria and statistical model were applied to epiGBS-D, epiGBS-R and WGBS filtered data if not stated otherwise. Furthermore, Pearson's Chi-square test ($\alpha = 0.05$) was used to test similarity in number of DMCs obtained between two methods (*chisq.test* funtion stats R package v3.6.2).

DIFFERENTIALLY METHYLATED REGIONS (DMRS)

In WGBS analyses is frequent to adopt a regional approach that considers the non-independence of DMCs that are close to each other within the genome, and combines them into regions (DMRs) to study DNA methylation differences between groups of samples (Laine et al., 2022). We call DMRs based on the following procedure: BedGraph files from EpiDiverse/WGBS pipeline were used as input for differential methylation analysis using the EpiDiverse/DMR pipeline v1.0 (https://github.com/EpiDiverse/dmr), each treatment group was compared to the control group and the three cytosine sequence contexts were analyzed separately. Briefly, DMRs were identified by metilene (https://www.bioinf.uni-leipzig.de/Software/metilene), using the following parameters: minimum read depth per position: 6; minimum cytosine number per DMR: 10; minimum distance between two different DMRs: 146 bp; per-group minimal non-missing data for estimating missing values: 0.8; adjusted p-value (Benjamini-Hochberg) to detect significant DMRs: 0.05. Only significant DMRs with methylation difference >10 % between control and treatment group were retained for analysis. Due to the short nature of epiGBS fragments (average length 207 nucleotides in the filtered data set), no formal DMR tests were performed.

STRUCTURAL ANNOTATION OF DMCS AND DMRS

We overlapped DMCs and DMRs from the former analyses with *P. nigra* cv. 'italica' (ENA project: PRJEB44889) genome to identify genomic features with differential methylation. We defined three genomic features: gene body, i.e. between transcriptional start and termination sites, the < 2 kb up-stream of transcriptional start (promoter region) and < 2 kb down-stream

of termination sites (downstream region). DMCs or DMRs located out of those three features were assigned to intergenic. Coordinates of DMC or DMRs were used to perform the BEDTools intersect command and a custom script for annotating each genomic feature including Transposable Elements (TE). Visualization of the distribution of DMCs for the treatment groups was carried out using custom R scripts (R Development Core Team, 2020).

FUNCTIONAL ANALYSIS OF GENES ASSOCIATED TO HERBIVORY-INDUCED DMRS

Each DMR was associated with its overlapping gene and/or with the closest gene (maximum 2kb upstream from TSS). Genes associated with either insect or artificial herbivory DMRs were subjected to gene ontology (GO) enrichment analysis. The gene set background (universe) was built with the closest *Arabidopsis* (*A. thaliana*) homologue of each *P. nigra* cv 'italica' gene, which was determined using BLAST best reciprocal hits (RBH) of the protein sequences (R package orthologr). Best hits were filtered by keeping alignments covering at least 60% of both *Arabidopsis* and *P. nigra* proteins, and minimum 60% similarity. *Arabidopsis* sequence proteins were extracted from phytozome V13, and functional annotations were retrieved from the PLAZA 5.0 dicots database (https://bioinformatics.psb.ugent.be/plaza/). GO enrichments were performed using clusterProfiler v4 (Wu et al., 2021). P-values were adjusted for multiple testing controlling the positive false discovery rate (q-value).

RESULTS

METHYLATION LEVELS ESTIMATED BY EPIGBS AND WGBS

As regards to methylation percentage recorded per cytosine, we found consistent outputs between the three techniques, with cytosines in CpG and CHG contexts showing a bimodal distribution with a much higher frequency in methylation levels < 25 %, while cytosines in CHH context showed unimodal distribution skewed towards low methylation and almost no case with methylation levels > 50 % (Fig 1). The average cytosine methylation level among all the poplar samples studied (N = 27) ranged from 8.1% to 28.2% according epiGBS-R, from 7.5% to 35.2% according to epiGBS-D, and from 8.0% to 38.79% according to WGBS.

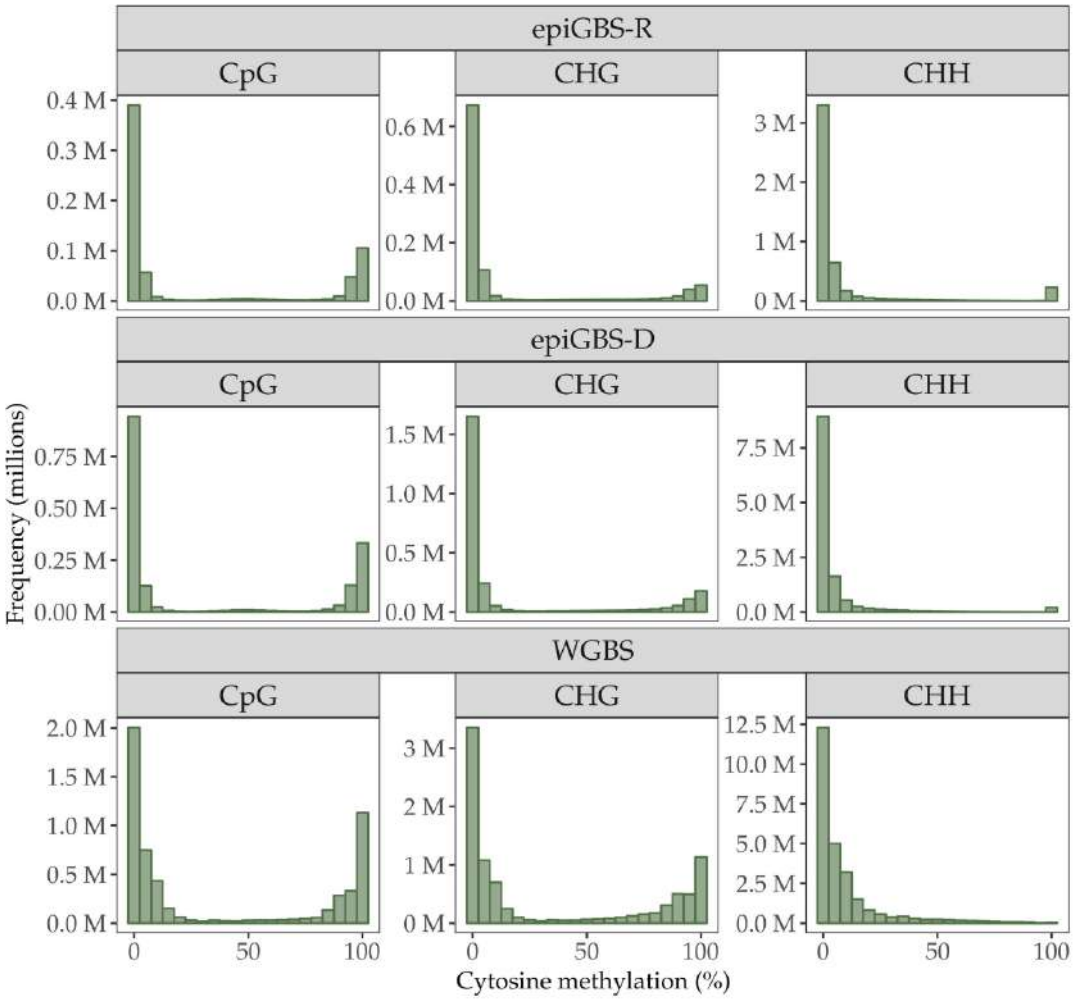

Fig 1. Histogram of DNA methylation percentage of cytosines captured by epiGBS-R, epiGBS-D and WGBS in CpG, CHG and CHH contexts. Methylation percentage was calculated as the mean value across all samples of per-cytosine methylation level.

Methylation percentage was higher in CpG, intermediate in CHG, and lower in CHH, regardless of the technique used for estimation. In epiGBS-R, methylation percentage across all samples averaged 27.8 ± 0.1%, 15.6 ± 0.1%, and 8.4 ± 0.1% for CpG, CHG, and CHH contexts, respectively. In epiGBS-D, average methylation obtained were similar to those in reference branch: 32.4 ± 0.1%, 19.0 ± 0.1%, and 5.7 ± 0.1% for CpG, CHG, and CHH context, respectively. In WGBS, average methylation percentage was 38.0 ± 0.5%, 34.9 ± 0.5%, and 9.5 ± 0.8% for CpG, CHG, and CHH contexts, respectively. The chi-squared tests conducted for each context indicated that average DNA methylation per context (i.e., interpreted as a relative proportion) was not statistically different among the three methods for CpG and CHH (Pearson's Chi-square test, CpG $X^2 = 1.61$, df = 2, $P = 0.45$ and CHH: $X^2 = 0.99$, df = 2, $P = 0.60$) but significantly different for the CHG context ($X^2 = 9.12$, df = 2, $P = 0.01$).

For each context, the linear model and ANOVA test applied to evaluate the effect of herbivory treatment and ortet indicated that average methylation percentage was significantly different among the three ortets for CpG and CHG according to epiGBS-R and epiGBS-D (Table 2). In CHH context, average methylation percentage was significantly different among levels of the herbivory treatment according to epiGBS-R and epiGBS-D (Table 2), methylation being lower in control plants (Fig 2). In WGBS, however, neither the ortet nor treatment had a significant effect on average DNA methylation recorded in any sequence contexts (Table 2). Given the similarities between epiGBS-D and epiGBS-R, only comparisons between WGBS and epiGBS-R are discussed further.

Table 2. Effect of ortet, herbivory treatment, and their interaction (Ortet × Herbivory) on genome wide average methylation percentage obtained by epiGBS-R, epiGBS-D and WGBS techniques for each of the three sequence contexts (CpG, CHG, and CHH).

		Source of variation					
		Ortet		Herbivory		Ortet × Herbivory	
Technique	**Sequence context**	*$F_{2, 18}$	***P***	$F_{2, 18}$	***P***	$F_{4, 18}$	***P***
epiGBS-R	**CpG**	**69.36**	**<0.0001**	1.50	0.25	0.56	0.69
	CHG	**3.35**	**0.05**	1.99	0.16	0.49	0.75
	CHH	0.47	0.63	**7.90**	**0.003**	1.22	0.34
epiGBS-D	**CpG**	**70.80**	**<0.0001**	1.26	0.30	0.63	0.64
	CHG	**4.72**	**0.02**	2.00	0.16	0.56	0.69
	CHH	0.38	0.68	**9.56**	**0.001**	1.46	0.26
WGBS	**CpG**	0.38	0.69	0.50	0.62	0.82	0.53
	CHG	0.55	0.59	0.23	0.8	0.72	0.59
	CHH	0.37	0.7	0.08	0.92	0.65	0.64

*F, degrees of freedom (subscript for "F") and P values are provided. Values are in bold when $P \leq 0.05$.

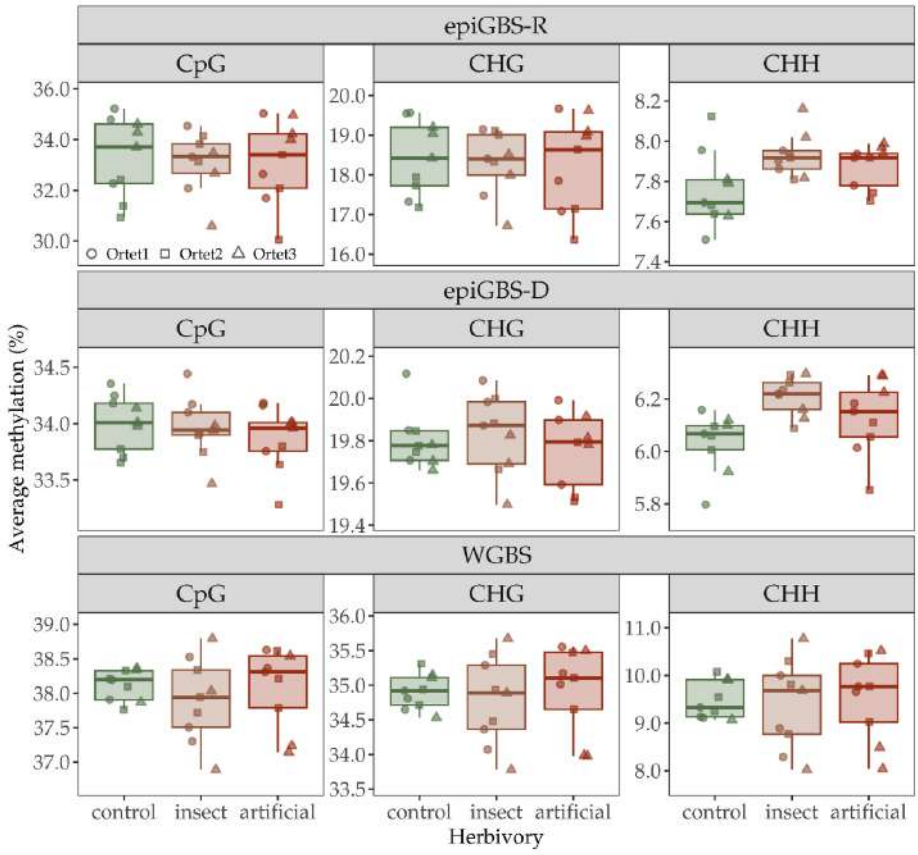

Fig 2. Average methylation levels (%) captured by epiGBS-R, epiGBS-D and WGBS in the three sequence contexts (CpG, CHG and CHH) in leaves of *P. nigra* cv. italica after insect and artificial herbivory treatment and in non-damaged controls. The boxplots depict medians ±1.5 x interquartile range of n = 9 replicates. Each dot denotes a replicate (ramet), and different symbols were used for the three ortets.

METHYLATION CHANGES INDUCED BY HERBIVORY DETECTED AS DMCS AND DMRS IN EPIGBS-R AND WGBS

In total, epiGBS-R captured 10,675 DMC, whereas WGBS was able to detect only 4,746 DMCs (S1 Table) for the two herbivory treatments combined. Such a difference between the two techniques was mainly due to a lack of captured DMCs at CHH context when analyzing WGBS data, likely due to reduced statistical power associated to multiple-testing correction requirements associated to the large number of CHH positions detected by WGBS (see Table 1). Still, the number of DMCs captured in the CpG and CHG contexts was similar in the two techniques (Pearson's Chi-square test: X^2= 0.02, df = 1, P = 0.88 for CpG context; X^2= 2.12, df = 1, P = 0.15 for CHG context). Furthermore, WGBS captured more DMCs in the CpG context than in the CHG context, but a similar number of DMCs were obtained in the two contexts with epiGBS (S1 Table). Overall, the number of DMCs identified in response to artificial herbivory was always greater than those induced by insect herbivory (2794 *vs* 1952 for epiGBS, and 5961 *vs* 4714 for WGBS, respectively, in artificial and insect herbivory).

Using the WGBS data, we identified a total of 1,057 DMRs, of which 500 DMRs (CG: 6, CHG: 29; CHH: 465 DMRs) were obtained for insect herbivory and 557 DMRs (CG: 9, CHG: 47; CHH 501) for artificial herbivory, and none was shared by the two treatments.

SIGNS OF METHYLATION SHIFTS AFTER HERBIVORY AND STRESS SPECIFICITY

In CpG context, the proportion of cytosines that shifted to a significantly lower methylation (hypo-methylated DMCs) and those that shifted to a significantly higher methylation (hyper-methylated DMCs) in

artificial herbivory was similar as detected either by epiGBS-R and WGBS (Pearson's Chi-square test: X^2= 1.3, df = 1, P = 0.24), indicating a higher frequency of hypo-methylation (Fig 3A). For the insect herbivory comparison, the relative frequency of hypo-methylated and hyper-DMCs was rather similar, regardless of the technique (Fig 3A). In CHG context, for both herbivory treatments the relative frequencies of hypo and hyper methylated loci varied depending on the applied technique, slightly more hyper-methylated DMCs were obtained by epiGBS and more hypo-methylated DMCs by WGBS and there appear to be significant differences between DMC numbers ($P < 0.05$; Fig 3A). Finally, in CHH context, a significantly higher number of hypermethylated DMCs was observed in response to insect and artificial herbivory treatments in epiGBS-R (Fig 3A; $P < 0.05$). As mentioned above, WGBS technique was largely inefficient for capturing DMCs in CHH context using the standard DMC calling parameters.

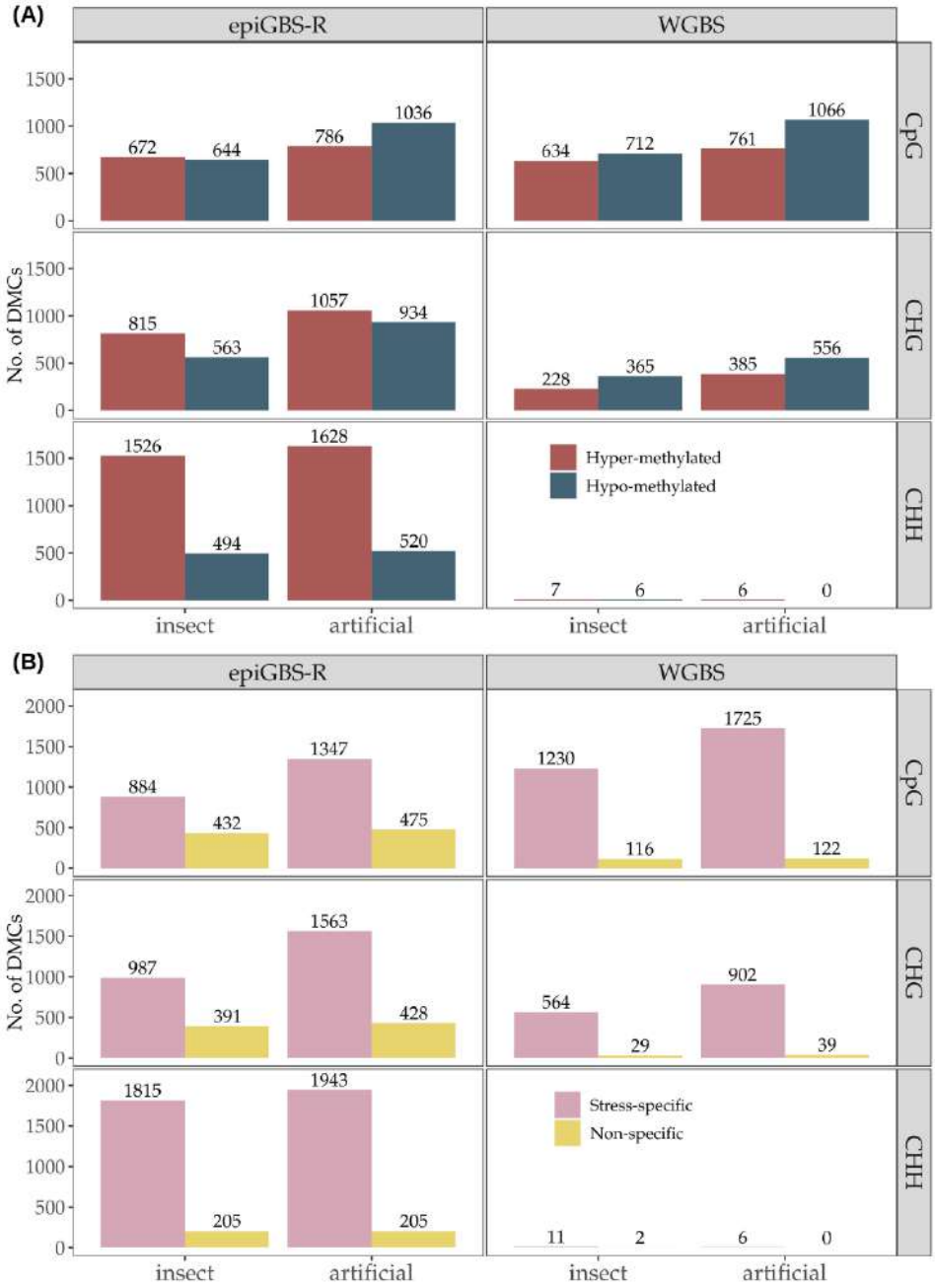

Fig 3. Differentially Methylated Cytosines (DMCs) in response to insect and artificial herbivory captured by epiGBS-R and WGBS in the three sequence contexts. DMCs were defined by a minimum coverage of 10X for epiGBS and 6X for WGBS, 10 % change in methylation percentage and q-value

<0.05. (A) Sign of methylation shifts after herbivory relative to the methylation status in controls, represented as hyper-methylated DMCs (in red) or hypo-methylated DMCs (in blue). (B) Specificity of the response to insect or artificial herbivory (stress specific DMCs, in pink; and non-specific DMCs, in yellow).

For either insect or artificial herbivory, treatment-specific DMCs (i.e., DMCs that only appear in one of the two herbivory treatments) were by far more abundant than non-specific ones (i.e., DMCs that appear in the two herbivory treatments) in all analyzed contexts, and this trend was found in both epiGBS-R and WGBS (Fig 3B). In particular, the relative frequency of stress specific DMCs was higher in WGBS (92.5% for CpG and 95.5% for CHG) than in epiGBS-R (71.1% for CpG, 75.6% for CHG, 90.1 % for CHH).

STRUCTURAL ANNOTATION OF DMCS AND DMRS INDUCED BY HERBIVORY

The overall results of structural annotation analyses of epiGBS-R data showed that DMCs induced by each of the two herbivory treatments in CpG and CHG contexts were present in all the distinct genome features identified. TEs that overlapped with the genomic features were indicated and we predominantly found DMCs within the gene body and the intergenic regions which were not TEs (Fig 4). However, in the CHH context much more DMCs were found in the intergenic regions and particularly overlapping with TEs. In WGBS data, a similar pattern was observed for CpG and CHG contexts and DMCs were predominantly found in gene bodies. As previously stated, no genomic feature was annotated in CHH context because of the failure to detect DMC with WGBS. In general, the amount of TEs detected by WGBS was lower than in epiGBS in most genomic features (Fig 4).

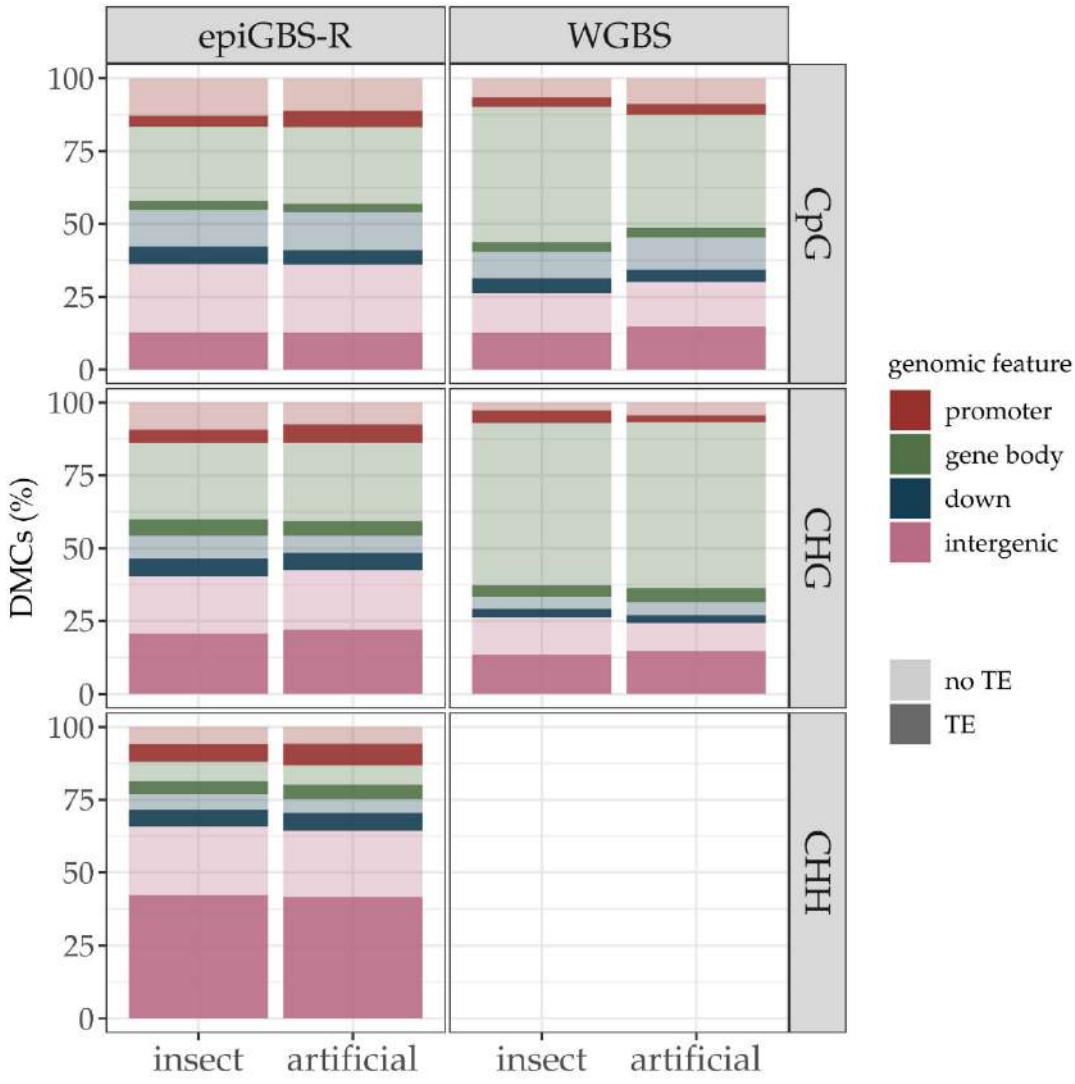

Fig 4. Genomic location of DMCs detected by epiGBS-R and WGBS in the three sequence contexts. DMCs mapped within genomic features were classified as being within the gene body (green), promoter (red), and 2 kb down-stream of termination sites (blue). DMCs located out of those regions were assigned to intergenic regions (purple). Information on location within Transposable Elements (TE) is also indicated, where darker colors indicate TE and lighter colors are no TE.

SIGNS OF METHYLATION SHIFTS AFTER HERBIVORY IN THE DIFFERENT GENIC REGIONS

In both CpG and CHG contexts, and in all the different genic features, the two techniques captured similar proportions of cytosines that shifted towards lower or higher methylation in response to herbivory (S2 Table). Specifically, in gene bodies not associated with TEs, a higher proportion of hypo-methylated DMCs were detected by epiGBS and WGBS in the two contexts, especially after artificial herbivory (Fig 5A). Also, the two techniques revealed that DMCs tended to be more hypo-methylated after artificial herbivory in intergenic regions that were not in TEs in CpG context. Finally, in CHG the two herbivory treatments tend to produce more hyper-methylation in intergenic regions both in TE and no TE, as detected mainly by epiGBS (Fig 5A).

In CHH context, DMCs captured by epiGBS in all genic features were predominantly hyper-methylated regardless of the type of herbivory experienced, being highest at intergenic regions in both TEs and non-TEs (Fig 5B). Consistent with these results, the largest number of DMRs detected by WGBS in response to the two herbivory treatments were also located at intergenic regions and they were predominantly hyper-methylated (Fig 5B), suggesting that the two techniques were able to detect similar biological response when properly analyzed.

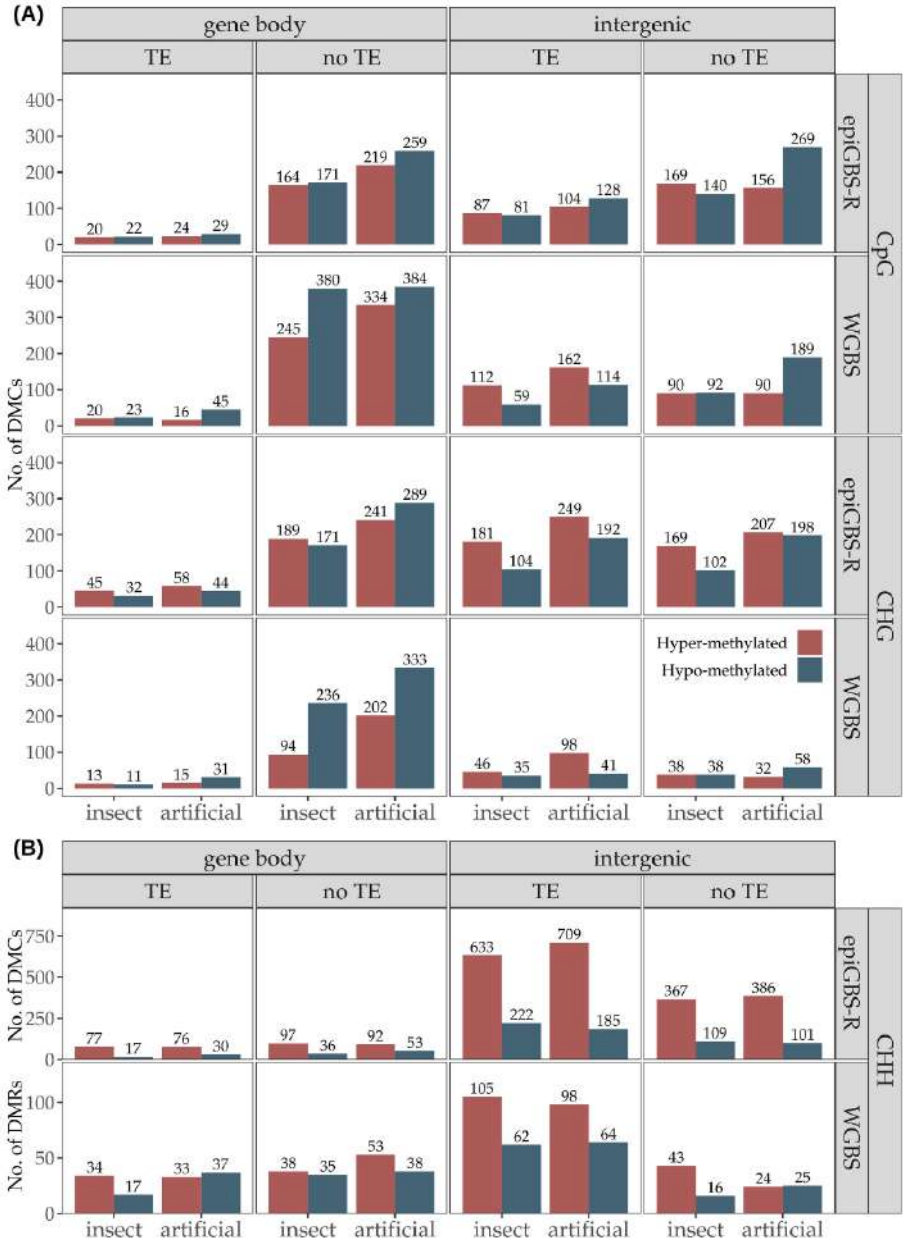

Fig 5. Methylation shifts in response to insect and artificial herbivory (relative to the methylation status in controls) captured by epiGBS-R and WGBS in the three different contexts that were located in gene bodies and in intergenic regions and their association with TE and non-TE regions. Hyper-methylated DMCs or DMRs were represented in red, and hypo-methylated in blue.

For WGBS due to a lack of captured DMCs at CHH context, bars represent DMRs. (A) DMCs obtained for CpG and CHG contexts. (B) DMCs obtained for epiGBS-R and DMRs for WGBS in CHH con-text. DMCs/DMRs had at least a 10 % difference in methylation compared to controls and q-value < 0.05.

FUNCTIONAL ASSOCIATION OF DIFFERENTIAL METHYLATION CHANGES OBSERVED IN RESPONSE TO HERBIVORY

None of the GO terms associated to DMCs or DMRs identified by epiGBS and WGBS showed enrichment after multiple testing correction (q-value < 0.05), however GO terms selected with uncorrected p-values < 0.05 suggested a functional association. In epiGBS, GO terms with uncorrected p < 0.05 indicated that insect herbivory-DMCs enriched genes in biological processes (S2A Fig) related to organelle organization (GO:0006996), phosphorylation (GO:0016310) and some other metabolic processes, while artificial herbivory-DMCs enriched genes in biological processes related to cellular response to stress (GO:0033554), and negative regulation to biological process (GO:0048519).

In WGBS, insect herbivory-DMRs enriched genes in biological processes (S2B Fig) related to defense response (GO:0006952), response to external biotic stimulus (GO:0043207) and defense response to other organism (GO:0098542), while artificial herbivory-DMRs enriched genes in biological processes (S2B Fig) related to response to abscisic acid (GO:0009737) and abscisic acid-activated signaling pathway (GO:0009738). However, neither of the treatments using epiGBS DMCs enriched GO terms in biological processes similar to WGBS. See additional S3 Table for a full list of enriched gene sets from the DMC and DMR analyses.

DISCUSSION

In this study, we analyzed DNA methylation in the genomes of Lombardy poplar clones that were either consumed by larvae of the gypsy moth (insect herbivory), hand-defoliated together with application of JA (artificial herbivory) or remained undamaged (control) growing under common conditions. We selected a clonal plant to obtain genetically uniform replicates that could facilitate the identification of independent epigenetic variation, *sensu* (Richards, 2006). Two single-base resolution methodologies were applied and their outputs compared, the WGBS approach which covered methylation in the entire genome and epiGBS which uses restriction enzymes to reduce the costs of sequencing by interrogating methylation in a portion of the genome (Paun et al., 2019). In the following paragraphs, we discuss the extent to which the genome-wide herbivory-induced methylation changes in undamaged leaves grown after treatment were comparable between the two types of herbivores assayed and how epiGBS and WGBS outputs detected these changes in terms of global methylation and differentially methylated loci.

OVERALL DNA METHYLATION CHANGES INDUCED BY HERBIVORY

Our WGBS analysis provided methylation information on > 116 million cytosines whereas epiGBS was able to analyze between 1.8 and 1.1 million cytosines depending on the use or not of the reference genome available (i.e., for epiGBS-R and epiGBS-D, respectively). In order to understand DNA methylation changes after herbivory, we paid attention to sequence context as cytosine methylation is introduced and maintained by different methyl-transferase and demethylase systems in the CpG, CHG and CHH contexts of DNA, that might respond differently to external stimuli

(Law & Jacobsen, 2010; Niederhuth et al., 2016). We found that DNA methylation was higher in CpG, intermediate in CHG, and lower in CHH, regardless of the technique used for estimation, with estimates at CHG being lower according to epiGBS. Furthermore, all techniques showed a bimodal distribution of cytosine methylation in the CpG context, with either unmethylated or methylated at very high levels, whereas the frequency of highly methylated positions was lower in the CHG and almost absent in CHH, similar to what is typically found in *Arabidopsis* (Cokus et al., 2008).

Variation in average methylation percentages after either artificial or insect herbivory estimated by epiGBS-R and epiGBS-D indicated a significant increased methylation in the CHH context, which is also the context more responsive to short-term stress in other plant species (Dowen et al., 2012; Lu et al., 2021). Methylation percentage in CpG and CHG did not change in response to herbivory but significant variation between the study ortets was observed in these two contexts, likely reflecting intrapopulation variance in methylomes of poplar trees retained after grafting (Perrin et al., 2020; Rodríguez et al., 2022). Methylation estimates based on WGBS did not significantly differ between herbivory treatments or ortets suggesting that the changes associated to the study factors, likely occurring in a small fraction of positions, were not reflected as an overall global methylation change with this technique. A higher sequencing depth in WGBS could circumvent the limitation observed here (Beck et al., 2022; Becker et al., 2011; Liu et al., 2012). Summing up, in our study epiGBS seemed to be efficient for identifying the global methylation changes associated to herbivory, suggesting that the genome sampling accomplished was representative to detect so, although differential methylation analyses should be more informative (see below).

DIFFERENTIAL METHYLATION ANALYSES: STRESS AND CONTEXT SPECIFIC CHANGES

The main purpose of applying single base resolution BS-seq methods is to capture differential methylation loci and their location according to genomic features that could be instrumental to understanding responses to a certain factor (H. Feng & Wu, 2019; Lister et al., 2008). These analyses require subsequent data filtering because reliable estimates of methylation for single evaluated positions need to be represented in most if not all samples (e.g., we required that every analyzed position was present in at least six out nine samples per group). In our filtered dataset, epiGBS and WGBS obtained comparable numbers of DMCs in CpG and CHG contexts for a change in methylation between control and treated plants larger than ten percent. In these two sequence contexts, all methods revealed that most captured DMCs were specific to one of the two herbivory treatments assayed, a result that contrasts somehow with a shared response in CpG and CHG contexts induced by pathogen infection and salicylic acid exposure in *A. thaliana* (Dowen et al., 2012). Artificial herbivory produced a higher number of DMCs than insect herbivory. Looking at the three sequence contexts we found that in CpG context, the proportion of cytosines that shifted to a significantly lower methylation (hypo-methylated DMCs) and those that shifted to a significantly higher methylation (hyper-methylated DMCs) were similar after insect herbivory whereas a higher number of hypo-methylated DMC were observed after artificial herbivory. In CHG context the ratio of hyper and hypo-methylated DMCs was not captured analogously by different methods, artificial and insect herbivory tended to show more hyper-methylated DMCs according to epiGBS and the opposite trend was obtained in WGBS. Previous studies showed that in rice, heavy metal treatment induced hypo-methylation

in CHG, whereas in *Arabidopsis*, hypo- and hyper-methylation modifications in CHG context were produced by an avirulent strain or the defense hormone Salicylic acid (Dowen et al., 2012; Ou et al., 2012).

Interestingly, in CHH context, epiGBS found the highest number of DMCs, with a clear prevalence of hyper-methylated DMCs, whereas WGBS obtained only a negligible number of DMCs, likely due to the reduced statistical power (because of multiple testing correction) to detect changes in methylation at positions that usually have very low methylation level when the number of evaluated positions is large (Beck et al., 2022; Becker et al., 2011; Liu et al., 2012).This pattern was observed in our study where 90,235,706 CHH sites were present in WGBS compared to the 877,942 in epiGBS raw data, but a minor percentage was retained by WGBS when only positions properly covered in all samples were selected (Table 1). For this reason, in WGBS, detection of differentially methylated regions (DMRs), *i.e.* contiguous stretches of DNA sequence in the genome that show differing levels of DNA methylation between groups of samples, has become a much frequent approach , provided that variation in DMR has been also associated with phenotypic plant variation (e.g.,(Becker et al., 2011; Cortijo et al., 2014)). When we applied the standard DMR identification method for WGBS, we found that they were most frequently found in CHH context and hyper-methylated DMRs were more frequent after either insect or artificial herbivory. Thus, in CHH context, the DMR output of WGBS showed similar relative frequency and sign than obtained by DMCs in epiGBS. A response characterised by hyper-methylated DMRs suggested that de novo methylation in CHH islands would be a suitable response associated to herbivory in the Lombardy poplar (see e.g., (Y.-Y. Zhang et al., 2013)). This is an interesting finding and may have implications for gene transcription as CHH hyper-

methylation in the proximity of a gene reduced the level of transcription of that particular gene in the apple tree (Perrin et al., 2020).

Overall, the epiGBS technique, which can be applied to any species in the absence of a reference genome at a lower cost per sample, was successful in demonstrating that methylation changes induced by insect and artificial herbivory occurred at distinct loci and indicated that increased methylation in the CHH context was the most frequently observed response. Thus, epiGBS could be particularly useful for non-model plant species and large experimental designs, such as those intended to search for species-specific epigenetic responses in plants typically damaged by a diverse array of antagonistic animals or pathogens, or the impact of multiple levels of abiotic conditions (e.g., temperature, water availability, and their combination) that could better simulate the different scenarios of climate change.

STRUCTURAL ANNOTATION AND FUNCTIONAL ASSOCIATION OF DMCS AND DMRS INDUCED BY HERBIVORY

Despite the fact that epiGBS usually covers a small percentage of the study genome (in our case 1.5%), our study found no apparent bias or preference for specific genomic features in the DMCs observed after artificial and insect herbivory in Lombardy poplar clones supporting that the method provided a sound genome-wise analysis (Gawehns, Postuma, Antro, et al., 2022). In particular, DMCs detected in CpG and CHG contexts were more frequently associated to hypo-methylation in gene bodies regardless of the method applied, an interesting finding provided that in *P. trichochocarpa* methylation in gene bodies had a more repressive effect on transcription than promoter methylation (Vining et al., 2012) and therefore the observed hypo-methylation might be associated with genes transcription. Conversely, the

most frequent change observed in CHH was hyper-methylation in TEs and intergenic regions. Such findings are consistent with response after pathogen infection in which increased CHH methylation levels along TE region were found in *A. thaliana* (Dowen et al., 2012). Additionally, hyper-methylation was suggested to be a general defense mechanism against pathogen stress in tobacco plants, and radiation-induced hyper-methylation in Scots pine was attributed to the activation of the Tes (Boyko et al., 2007; Volkova et al., 2018).

As a further step to better understand the potential consequences of methylation changes observed, we conducted gene enrichment analyses associated to DMRs from WGBS and DMCs from epiGBS to compare their outputs, provided there is currently no unified method for performing enrichment analyses of DMRs from both approaches. Gene enrichment analysis from WGBS-DMR revealed that most changes associated with herbivory were related to the Gene Ontology (GO) category associated to Biological Processes, but enrichment in specific GO categories differed between insect herbivory and artificial herbivory. Insect herbivory was more related to responses to biotic stimuli, defense responses and immune system processes, whereas changes associated with artificial herbivory were more related to abscisic acid stimulus, ligase and cell cycle process, and mRNA processing. The genes associated to epiGBS-DMCs were equally associated to biological process, molecular function and cellular components categories, mainly related to catabolic processes or organelle organization within the cell. Therefore, epiGBS is useful for understanding the localization and direction of differential methylation, but it does not directly reveal specific functional response in genes associated to DMCs, a constraint that is an inherent

limitation of epiGBS (and RRBS in general) because the majority of genes in the genome are not included in the fraction of the genome analyzed.

CONCLUSION

To sum up, we found that epiGBS offered reliable insight about methylation changes in DNA of Lombardy poplar clones experiencing insect and artificial herbivory. The results offered by epiGBS and WGBS were consistent as regards (i) the context dependent response, mainly associated to increased methylation in CHH, (ii) the specificity of the response elicited by insect and artificial herbivory indicated by the few shared DMCs, and (iii) the structural annotation of those changes, mainly associated to TEs and intergenic regions for CHH, and to gene bodies and their flanking regions in CpG and CHG. Thus, epiGBS succeeded to characterize global, genome-wide methylation changes in response to a certain stress, being particularly useful for investigating species lacking a reference genome, whereas WGBS performed better in the functional analysis. The functional interpretation (at the level of GO term enrichment) of the observed methylation changes remained unclear and additional transcriptome analyses might be instrumental to characterize the epigenetic regulation of stress-specific responses in non-model plants with limited genomic resources.

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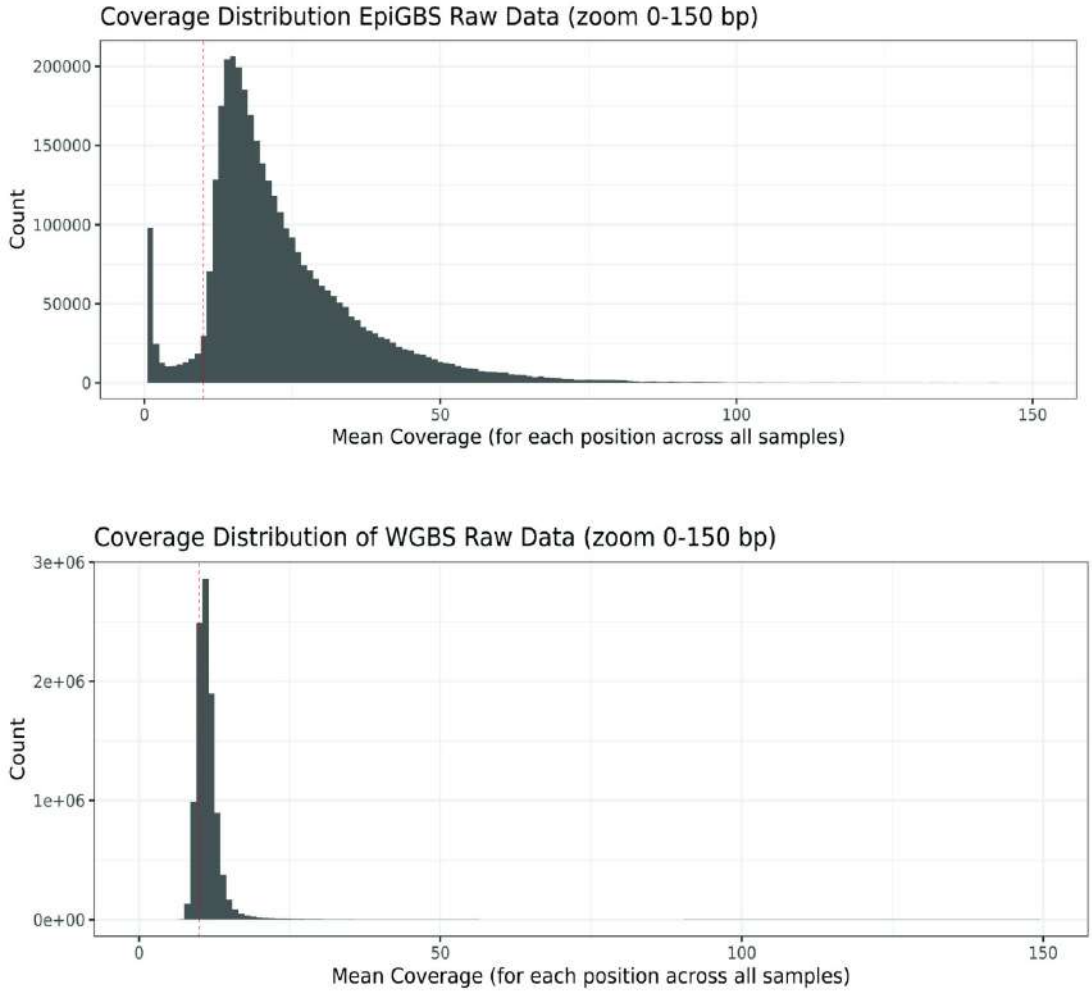
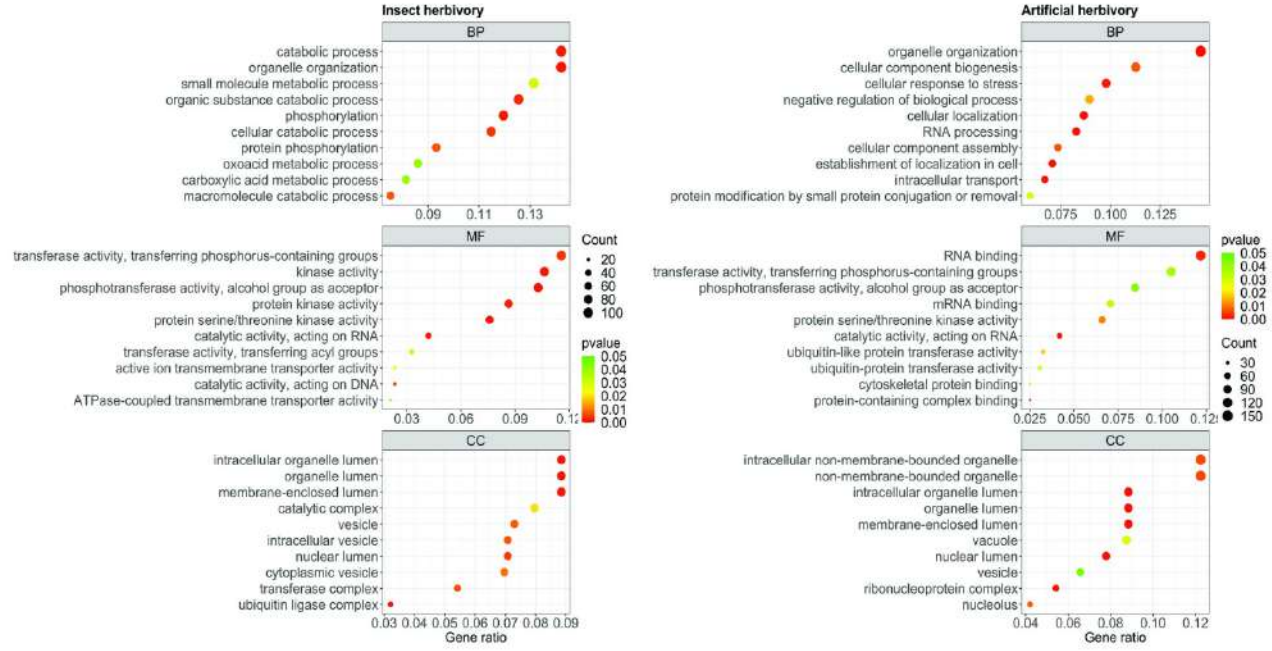
SUPPORTING INFORMATION

S1 Fig. Mean coverage distribution for each position across all samples obtained with epiGBS and WGBS.

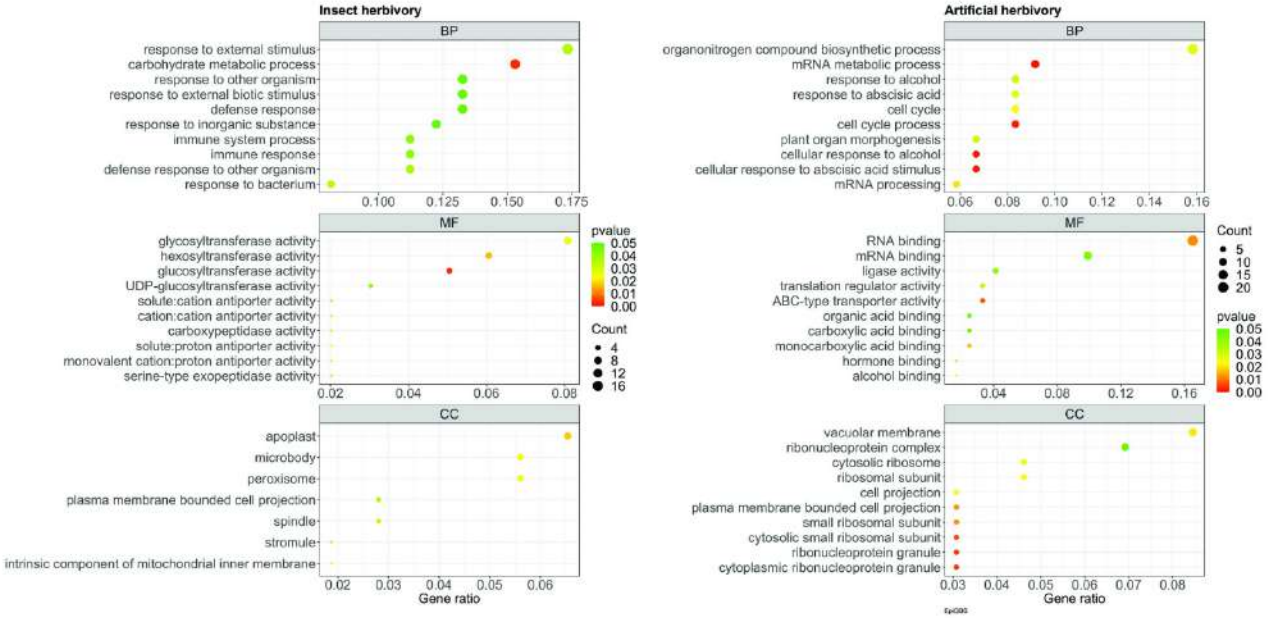


S2 Fig. Gene ontology (GO) clustering and selected significantly enriched GO terms of DMCs and DMR-associated genes.'

(A) epiGBS



(B) WGBS



S1 Table. Total number of differentially methylated cytosines for insect and artificial treated plants captured by WGBS and epiGBS-R (reference branch) in the three cytosine contexts. In all the comparisons the filtering criteria was q-value<0.05 and methylation difference >10%.

	Context	Insect		Artificial	
		#	%	#	%
epiGBS-R	**CpG**	1316	27.9	1822	30.6
	CHG	1378	29.2	1991	33.4
	CHH	2020	42.9	2148	36.0
WGBS	**CpG**	1346	69.0	1847	66.1
	CHG	593	30.4	941	34.0
	CHH	13	0.6	6	0.9

S2 Table. Total number of differentially methylated cytosines (DMC) captured by epiGBS-R (reference branch) and WGBS in the three sequence contexts (CpG, CHG and CHH) in each genomic feature (promoter, down-stream of transcriptional sites, gene body and intergenic region), within transposable elements (TE) or outside (no TE) also indicated. DMCs were defined by a minimum coverage of 10X for epiGBS and 6X for WGBS, 10 % change in methylation percentage and q-value <0.05. Higher and lower methylation shifts after herbivory relative to the methylation status in controls are represented respectively as hyper-methylated and hypo-methylated DMCs.

Technique	Context	Herbivory	Genomic feature	TE		no TE	
				hyper	hypo	hyper	hypo
epiGBS-R	CpG	insect	promoter	22	27	79	90
			down	46	33	85	80
			gene body	20	22	164	171
			intergenic	87	81	169	140
		artificial	promoter	32	70	84	120
			down	52	39	115	122
			gene body	24	29	219	259
			intergenic	104	128	156	269
	CHG	insect	promoter	44	22	66	61
			down	56	29	65	42
			gene body	45	32	189	171
			intergenic	181	104	169	102
		artificial	promoter	73	56	90	59
			down	83	36	56	60

			gene body	58	44	241	289
			intergenic	249	192	207	198
	CHH	insect	promoter	100	24	84	34
			down	92	24	76	28
			gene body	77	17	97	36
			intergenic	633	222	367	109
		artificial	promoter	123	37	84	38
			down	88	45	70	31
			gene body	76	30	92	53
			intergenic	709	185	386	101
WGBS	CpG	insect	promoter	21	23	44	45
			down	50	20	52	70
			gene body	20	23	245	380
			intergenic	112	59	90	92
		artificial	promoter	17	50	60	103
			down	24	54	58	147
			gene body	16	45	334	384
			intergenic	162	114	90	189
	CHG	insect	promoter	8	18	7	9
			down	11	5	11	13
			gene body	13	11	94	236
			intergenic	46	35	38	38
		artificial	promoter	6	17	16	25
			down	7	19	9	32
			gene body	15	31	202	333
			intergenic	98	41	32	58
	CHH	insect	promoter	2	2	2	1
			intergenic	3	3	-	-
		artificial	intergenic	6	-		

S3 Table. Full list of enriched gene sets from the DMC and DMR analyses.

stress	technique	ID	Description	pvalue	p.adjust	qvalue	geneID	bgsource	PN_annoated_genes	foldEnrichme
Insect	WGBS	GO:0005975	carbohydrate metabolic process	0.00350815	0.00350815	0.5462227	AT3G04880.	BP	augustus_masked-scaffold_28-processed-	2.17055277
Insect	WGBS	GO:0010087	phloem or xylem histogenesis	0.00706338	0.00706338	0.5462227	AT5G19530.	BP	snap_masked-scaffold_25-processed-gen	5.27692537
Insect	WGBS	GO:0019853	L-ascorbic acid biosynthetic process	0.00772603	0.00772603	0.5462227	AT4G26850.	BP	augustus_masked-scaffold_43-processed-	15.0612245
Insect	WGBS	GO:1901698	response to nitrogen compound	0.00949213	0.00949213	0.5462227	AT2G14610.	BP	maker-scaffold_9-snap_gene-28.88-mRNA	3.32640541
Insect	WGBS	GO:0046777	protein autophosphorylation	0.01127929	0.01127929	0.5462227	AT1G77720.	BP	maker-scaffold_1-snap_gene-30.65-mRNA	3.71882086
Insect	WGBS	GO:0010167	response to nitrate	0.01191789	0.01191789	0.5462227	AT1G53310.	BP	maker-scaffold_38-augustus_gene-3.35-m	12.0489796
Insect	WGBS	GO:0015977	carbon fixation	0.01191789	0.01191789	0.5462227	AT1G53310.	BP	maker-scaffold_38-augustus_gene-3.35-m	12.0489796
Insect	WGBS	GO:0019852	L-ascorbic acid metabolic process	0.01191789	0.01191789	0.5462227	AT4G26850.	BP	augustus_masked-scaffold_43-processed-	12.0489796
Insect	WGBS	GO:0008360	regulation of cell shape	0.01689418	0.01689418	0.5462227	AT5G49430.	BP	maker-scaffold_11-snap_gene-33.99-mRN	10.0408163
Insect	WGBS	GO:0006098	pentose-phosphate shunt	0.01871771	0.01871771	0.5462227	AT3G02360.	BP	maker-scaffold_11-snap_gene-35.102-mR	9.51235231
Insect	WGBS	GO:0006073	cellular glucan metabolic process	0.02020906	0.02020906	0.5462227	AT1G68560.	BP	maker-scaffold_11-snap_gene-38.72-mRN	3.20451585
Insect	WGBS	GO:0044042	glucan metabolic process	0.02048493	0.02048493	0.5462227	AT1G68560.	BP	maker-scaffold_11-snap_gene-38.72-mRN	3.19319247
Insect	WGBS	GO:0006813	potassium ion transport	0.02102511	0.02102511	0.5462227	AT5G37060.	BP	maker-scaffold_9-augustus_gene-25.84-m	5.11513285
Insect	WGBS	GO:0010200	response to chitin	0.02154672	0.02154672	0.5462227	AT3G05200.	BP	augustus_masked-scaffold_44-processed-	5.06732787
Insect	WGBS	GO:0006740	NADPH regeneration	0.02160069	0.02160069	0.5462227	AT3G02360.	BP	maker-scaffold_11-snap_gene-35.102-mR	8.81632653
Insect	WGBS	GO:0006885	regulation of pH	0.0226001	0.0226001	0.5462227	AT5G37060.	BP	maker-scaffold_9-augustus_gene-25.84-m	8.60641399
Insect	WGBS	GO:0007029	endoplasmic reticulum organization	0.02465512	0.02465512	0.5462227	AT1G64090.	BP	maker-scaffold_6-snap_gene-20.94-mRNA	8.21521336
Insect	WGBS	GO:0006935	chemotaxis	0.02571027	0.02571027	0.5462227	AT4G08850.	BP	maker-scaffold_540-augustus_gene-0.7-m	8.03265306
Insect	WGBS	GO:0010183	pollen tube guidance	0.02571027	0.02571027	0.5462227	AT4G08850.	BP	maker-scaffold_540-augustus_gene-0.7-m	8.03265306
Insect	WGBS	GO:0042330	taxis	0.02571027	0.02571027	0.5462227	AT4G08850.	BP	maker-scaffold_540-augustus_gene-0.7-m	8.03265306
Insect	WGBS	GO:0050918	positive chemotaxis	0.02571027	0.02571027	0.5462227	AT4G08850.	BP	maker-scaffold_540-augustus_gene-0.7-m	8.03265306
Insect	WGBS	GO:0051156	glucose 6-phosphate metabolic process	0.02571027	0.02571027	0.5462227	AT3G02360.	BP	maker-scaffold_11-snap_gene-35.102-mR	8.03265306
Insect	WGBS	GO:0016051	carbohydrate biosynthetic process	0.02768015	0.02768015	0.5462227	AT4G26850.	BP	augustus_masked-scaffold_43-processed-	2.61303172
Insect	WGBS	GO:0006275	regulation of DNA replication	0.0278747	0.0278747	0.5462227	AT5G04590.	BP	maker-scaffold_9-augustus_gene-14.95-m	7.69083804
Insect	WGBS	GO:0016052	carbohydrate catabolic process	0.02888772	0.02888772	0.5462227	AT2G29560.	BP	maker-scaffold_7-snap_gene-21.113-mRN	2.91507571
Insect	WGBS	GO:0040011	locomotion	0.03010977	0.03010977	0.5462227	AT4G08850.	BP	maker-scaffold_540-augustus_gene-0.7-m	7.37692628
Insect	WGBS	GO:0052545	callose localization	0.03010977	0.03010977	0.5462227	AT4G26850.	BP	augustus_masked-scaffold_43-processed-	7.37692628
Insect	WGBS	GO:0006487	protein N-linked glycosylation	0.03125324	0.03125324	0.5462227	AT5G38460.	BP	maker-scaffold_57-augustus_gene-2.52-m	7.22938776
Insect	WGBS	GO:0009617	response to bacterium	0.03421463	0.03421463	0.5462227	AT1G64090.	BP	maker-scaffold_6-snap_gene-20.94-mRNA	2.13256276
Insect	WGBS	GO:0009605	response to external stimulus	0.03469922	0.03469922	0.5462227	AT4G08850.	BP	maker-scaffold_540-augustus_gene-0.7-m	1.59526988
Insect	WGBS	GO:0033037	polysaccharide localization	0.03478474	0.03478474	0.5462227	AT4G26850.	BP	augustus_masked-scaffold_43-processed-	6.82017713
Insect	WGBS	GO:0098542	defense response to other organism	0.03719868	0.03719868	0.5462227	AT2G14610.	BP	maker-scaffold_9-snap_gene-28.88-mRNA	1.8323333
Insect	WGBS	GO:0006739	NADP metabolic process	0.03846334	0.03846334	0.5462227	AT3G02360.	BP	maker-scaffold_11-snap_gene-35.102-mR	6.4548105
Insect	WGBS	GO:0006099	tricarboxylic acid cycle	0.03972124	0.03972124	0.5462227	AT1G53310.	BP	maker-scaffold_38-augustus_gene-3.35-m	6.34156821
Insect	WGBS	GO:0033043	regulation of organelle organization	0.04044666	0.04044666	0.5462227	AT1G77720.	BP	maker-scaffold_1-snap_gene-30.65-mRNA	3.10274153
Insect	WGBS	GO:0010411	xyloglucan metabolic process	0.04099464	0.04099464	0.5462227	AT1G68560.	BP	maker-scaffold_11-snap_gene-38.72-mRN	6.23223082
Insect	WGBS	GO:0006955	immune response	0.04180852	0.04180852	0.5462227	AT2G14610.	BP	maker-scaffold_9-snap_gene-28.88-mRNA	1.79754216
Insect	WGBS	GO:0002376	immune system process	0.04272996	0.04272996	0.5462227	AT2G14610.	BP	maker-scaffold_9-snap_gene-28.88-mRNA	1.79106453
Insect	WGBS	GO:0010089	xylem development	0.04758697	0.04758697	0.5462227	AT5G19530.	BP	snap_masked-scaffold_25-processed-gen	5.73760933
Insect	WGBS	GO:0006952	defense response	0.04883474	0.04883474	0.5462227	AT2G14610.	BP	maker-scaffold_9-snap_gene-28.88-mRNA	1.65577944
Insect	WGBS	GO:0010035	response to inorganic substance	0.04908858	0.04908858	0.5462227	AT2G14610.	BP	maker-scaffold_9-snap_gene-28.88-mRNA	1.69836831
Insect	WGBS	GO:0043207	response to external biotic stimulus	0.04929028	0.04929028	0.5462227	AT2G14610.	BP	maker-scaffold_9-snap_gene-28.88-mRNA	1.65344899
Insect	WGBS	GO:0051707	response to other organism	0.04929028	0.04929028	0.5462227	AT2G14610.	BP	maker-scaffold_9-snap_gene-28.88-mRNA	1.65344899
Insect	WGBS	GO:0046527	glucosyltransferase activity	0.00327864	0.00327864	0.479981682	AT4G28850.	MF	maker-scaffold_13-augustus_gene-23.66-	5.01228501
Insect	WGBS	GO:0015385	sodium:proton antiporter activity	0.00416279	0.00416279	0.479981682	AT5G37060.	MF	maker-scaffold_9-augustus_gene-25.84-m	20.6060606
Insect	WGBS	GO:0051139	metal ion:proton antiporter activity	0.0092538	0.0092538	0.55301304	AT5G37060.	MF	maker-scaffold_9-augustus_gene-25.84-m	13.7373737
Insect	WGBS	GO:0004185	serine-type carboxypeptidase activity	0.01363184	0.01363184	0.55301304	AT3G63470.	MF	maker-scaffold_9-augustus_gene-16.72-m	11.2396694

stress	technique	ID	Description	pvalue	p.adjust	qvalue	geneID	bgsource	PN_annoated_genes	foldEnrichme
Insect	WGBS	GO:0015081	sodium ion transmembrane transporter	0.01443345	0.01443345	0.55301304	AT5G37060.	MF	maker-scaffold_9-augustus-gene-25.84-m	10.9090909
Insect	WGBS	GO:0016758	hexosyltransferase activity	0.01652949	0.01652949	0.55301304	AT4G28850.	MF	maker-scaffold_13-augustus-gene-23.66-r	2.94372294
Insect	WGBS	GO:0070008	serine-type exopeptidase activity	0.01873568	0.01873568	0.55301304	AT3G63470.	MF	maker-scaffold_9-augustus-gene-16.72-m	9.51048951
Insect	WGBS	GO:0005451	monovalent cation:proton antiporter act	0.02154409	0.02154409	0.55301304	AT5G37060.	MF	maker-scaffold_9-augustus-gene-25.84-m	8.83116883
Insect	WGBS	GO:0015299	solute:proton antiporter activity	0.02251674	0.02251674	0.55301304	AT5G37060.	MF	maker-scaffold_9-augustus-gene-25.84-m	8.62579281
Insect	WGBS	GO:0016757	glycosyltransferase activity	0.02398915	0.02398915	0.55301304	AT1G19360.	MF	maker-scaffold_7-augustus-gene-43.98-m	2.28603446
Insect	WGBS	GO:0004180	carboxypeptidase activity	0.02872063	0.02872063	0.56210628	AT3G63470.	MF	maker-scaffold_9-augustus-gene-16.72-m	7.56957328
Insect	WGBS	GO:0015491	cation:cation antiporter activity	0.02981397	0.02981397	0.56210628	AT5G37060.	MF	maker-scaffold_9-augustus-gene-25.84-m	7.41818182
Insect	WGBS	GO:0015298	solute:cation antiporter activity	0.03671135	0.03671135	0.56210628	AT5G37060.	MF	maker-scaffold_9-augustus-gene-25.84-m	6.62337662
Insect	WGBS	GO:0035251	UDP-glucosyltransferase activity	0.04150863	0.04150863	0.56210628	AT4G26850.	MF	augustus_masked-scaffold_43-processed-	3.91805378
Insect	WGBS	GO:0048046	apoplast	0.01759437	0.01759437	0.64267349	AT2G14610.	CC	maker-scaffold_9-snap-gene-28.88-mRNA	2.62651942
Insect	WGBS	GO:0005777	peroxisome	0.02488323	0.02488323	0.64267349	AT2G29560.	CC	maker-scaffold_7-snap-gene-21.113-mRN	2.68252197
Insect	WGBS	GO:0042579	microbody	0.02488323	0.02488323	0.64267349	AT2G29560.	CC	maker-scaffold_7-snap-gene-21.113-mRN	2.68252197
Insect	WGBS	GO:0031304	intrinsic component of mitochondrial inr	0.02707663	0.02707663	0.64267349	AT1G09575.	CC	maker-scaffold_98-augustus-gene-0.101-r	7.81430313
Insect	WGBS	GO:0005819	spindle	0.0333096	0.0333096	0.64267349	AT5G48520.	CC	maker-scaffold_54-snap-gene-5.29-mRNA	4.27926124
Insect	WGBS	GO:0010319	stromule	0.03515924	0.03515924	0.64267349	AT5G04590.	CC	maker-scaffold_9-augustus-gene-14.95-m	6.78222536
Insect	WGBS	GO:0120025	plasma membrane bounded cell projecti	0.03673836	0.03673836	0.64267349	AT4G08850.	CC	maker-scaffold_540-augustus-gene-0.7-m	4.11593066
Artificial	WGBS	GO:0016071	mRNA metabolic process	0.00112502	0.00112502	0.41513487	AT4G20910.	BP	maker-scaffold_22-snap-gene-28.128-mR	3.00110906
Artificial	WGBS	GO:0010257	NADH dehydrogenase complex assembly	0.00142662	0.00142662	0.41513487	AT1G76060.	BP	augustus_masked-scaffold_1-processed-g	13.4181818
Artificial	WGBS	GO:0071215	cellular response to abscisic acid stimulu	0.00194782	0.00194782	0.41513487	AT1G52920.	BP	maker-scaffold_10-snap-gene-38.56-mRN	3.54594595
Artificial	WGBS	GO:0097306	cellular response to alcohol	0.00194782	0.00194782	0.41513487	AT1G52920.	BP	maker-scaffold_10-snap-gene-38.56-mRN	3.54594595
Artificial	WGBS	GO:0022402	cell cycle process	0.00213408	0.00213408	0.41513487	AT5G47690.	BP	maker-scaffold_4-snap-gene-69.82-mRNA	2.952
Artificial	WGBS	GO:0009738	abscisic acid-activated signaling pathwa	0.00451364	0.00451364	0.51187875	AT1G52920.	BP	maker-scaffold_10-snap-gene-38.56-mRN	3.42119205
Artificial	WGBS	GO:0046620	regulation of organ growth	0.00513361	0.00513361	0.51187875	AT5G27780.	BP	maker-scaffold_16-snap-gene-18.79-mRN	18.45
Artificial	WGBS	GO:0080156	mitochondrial mRNA modification	0.00962393	0.00962393	0.51187875	AT3G12770.	BP	maker-scaffold_78-snap-gene-1.119-mRN	13.4181818
Artificial	WGBS	GO:0032981	mitochondrial respiratory chain complex	0.01049416	0.01049416	0.51187875	AT1G76060.	BP	augustus_masked-scaffold_1-processed-g	12.8347826
Artificial	WGBS	GO:0071577	zinc ion transmembrane transport	0.01049416	0.01049416	0.51187875	AT3G08650.	BP	maker-scaffold_45-snap-gene-1.87-mRNA	12.8347826
Artificial	WGBS	GO:0034605	cellular response to heat	0.01103985	0.01103985	0.51187875	AT3G22840.	BP	maker-scaffold_25-augustus-gene-5.102-r	6.51176471
Artificial	WGBS	GO:0015979	photosynthesis	0.01187338	0.01187338	0.51187875	AT3G04790.	BP	augustus_masked-scaffold_28-processed-	3.17419355
Artificial	WGBS	GO:1900864	mitochondrial RNA modification	0.0123346	0.0123346	0.51187875	AT3G12770.	BP	maker-scaffold_78-snap-gene-1.119-mRN	11.808
Artificial	WGBS	GO:0048509	regulation of meristem development	0.01288453	0.01288453	0.51187875	AT4G09510.	BP	maker-scaffold_30-augustus-gene-11.76-r	6.15
Artificial	WGBS	GO:0006829	zinc ion transport	0.01330385	0.01330385	0.51187875	AT3G08650.	BP	maker-scaffold_45-snap-gene-1.87-mRNA	11.3538462
Artificial	WGBS	GO:0007062	sister chromatid cohesion	0.01533799	0.01533799	0.51187875	AT5G47690.	BP	maker-scaffold_4-snap-gene-69.82-mRNA	10.5428571
Artificial	WGBS	GO:0035265	organ growth	0.01640195	0.01640195	0.51187875	AT5G27780.	BP	maker-scaffold_16-snap-gene-18.79-mRN	10.1793103
Artificial	WGBS	GO:0035435	phosphate ion transmembrane transport	0.01749657	0.01749657	0.51187875	AT5G14040.	BP	maker-scaffold_40-augustus-gene-4.33-m	9.84
Artificial	WGBS	GO:0051607	defense response to virus	0.01764396	0.01764396	0.51187875	AT4G20910.	BP	maker-scaffold_22-snap-gene-28.128-mR	5.46666667
Artificial	WGBS	GO:0140546	defense response to symbiont	0.01764396	0.01764396	0.51187875	AT4G20910.	BP	maker-scaffold_22-snap-gene-28.128-mR	5.46666667
Artificial	WGBS	GO:0006397	mRNA processing	0.01989469	0.01989469	0.51187875	AT2G13540.	BP	maker-scaffold_7-snap-gene-23.112-mRN	2.56377171
Artificial	WGBS	GO:0031399	regulation of protein modification proce	0.02124157	0.02124157	0.51187875	AT2G25760.	BP	maker-scaffold_23-augustus-gene-10.33-r	3.16738197
Artificial	WGBS	GO:0000911	cytokinesis by cell plate formation	0.02259211	0.02259211	0.51187875	AT3G48860.	BP	augustus_masked-scaffold_19-processed-	4.9752809
Artificial	WGBS	GO:0007049	cell cycle	0.02283938	0.02283938	0.51187875	AT5G47690.	BP	maker-scaffold_4-snap-gene-69.82-mRNA	2.07303371
Artificial	WGBS	GO:0034293	sexual sporulation	0.02341347	0.02341347	0.51187875	AT5G47690.	BP	maker-scaffold_4-snap-gene-69.82-mRNA	8.43428571
Artificial	WGBS	GO:0043934	sporulation	0.02341347	0.02341347	0.51187875	AT5G47690.	BP	maker-scaffold_4-snap-gene-69.82-mRNA	8.43428571
Artificial	WGBS	GO:0048236	plant-type sporogenesis	0.02341347	0.02341347	0.51187875	AT5G47690.	BP	maker-scaffold_4-snap-gene-69.82-mRNA	8.43428571
Artificial	WGBS	GO:0033108	mitochondrial respiratory chain complex	0.02468252	0.02468252	0.51187875	AT1G76060.	BP	augustus_masked-scaffold_1-processed-g	8.2
Artificial	WGBS	GO:1901566	organonitrogen compound biosynthetic p	0.026928	0.026928	0.51187875	AT1G43170.	BP	augustus_masked-scaffold_1-processed-g	1.59794872
Artificial	WGBS	GO:0009737	response to abscisic acid	0.02698789	0.02698789	0.51187875	AT2G13540.	BP	maker-scaffold_7-snap-gene-23.112-mRN	2.01639344
Artificial	WGBS	GO:0002183	cytoplasmic translational initiation	0.02730284	0.02730284	0.51187875	AT1G71350.	BP	maker-scaffold_29-augustus-gene-4.35-m	7.76842105
Artificial	WGBS	GO:0016556	mRNA modification	0.02730284	0.02730284	0.51187875	AT3G12770.	BP	maker-scaffold_78-snap-gene-1.119-mRN	7.76842105
Artificial	WGBS	GO:0048506	regulation of timing of meristematic pha	0.02730284	0.02730284	0.51187875	AT4G09510.	BP	maker-scaffold_30-augustus-gene-11.76-r	7.76842105

stress	technique	ID	Description	pvalue	p.adjust	qvalue	geneID	bgsource	PN_annoated_genes	foldEnrichme
Artificial	WGBS	GO:0048510	regulation of timing of transition from v	0.02730284	0.02730284	0.51187875	AT4G09510.4	BP	maker-scaffold_30-augustus-gene-11.76-	7.76842105
Artificial	WGBS	GO:1903311	regulation of mRNA metabolic process	0.02747888	0.02747888	0.51187875	AT4G11970.1	BP	maker-scaffold_6-snap-gene-27.103-mRN	4.6125
Artificial	WGBS	GO:0044270	cellular nitrogen compound catabolic pro	0.02982232	0.02982232	0.51187875	AT2G13540.1	BP	maker-scaffold_7-snap-gene-23.112-mRN	2.89411765
Artificial	WGBS	GO:0019220	regulation of phosphate metabolic proce	0.0301434	0.0301434	0.51187875	AT2G25760.1	BP	maker-scaffold_23-augustus-gene-10.33-	3.41271676
Artificial	WGBS	GO:1905392	plant organ morphogenesis	0.03056118	0.03056118	0.51187875	AT4G20910.1	BP	maker-scaffold_22-snap-gene-28.128-mR	2.18666667
Artificial	WGBS	GO:0097305	response to alcohol	0.03067736	0.03067736	0.51187875	AT2G13540.1	BP	maker-scaffold_7-snap-gene-23.112-mRN	1.97326203
Artificial	WGBS	GO:0035196	production of miRNAs involved in gene s	0.03143253	0.03143253	0.51187875	AT4G20910.1	BP	maker-scaffold_22-snap-gene-28.128-mR	7.2
Artificial	WGBS	GO:0051174	regulation of phosphorus metabolic proc	0.03182034	0.03182034	0.51187875	AT2G25760.1	BP	maker-scaffold_23-augustus-gene-10.33-	3.35454546
Artificial	WGBS	GO:0006817	phosphate ion transport	0.03431366	0.03431366	0.51187875	AT5G14040.4	BP	maker-scaffold_40-augustus-gene-4.33-m	6.86511628
Artificial	WGBS	GO:0001932	regulation of protein phosphorylation	0.03620291	0.03620291	0.51187875	AT2G25760.1	BP	maker-scaffold_23-augustus-gene-10.33-	4.13831776
Artificial	WGBS	GO:0006935	chemotaxis	0.03729344	0.03729344	0.51187875	AT4G08850.1	BP	maker-scaffold_34-snap-gene-12.110-mR	6.56
Artificial	WGBS	GO:0010183	pollen tube guidance	0.03729344	0.03729344	0.51187875	AT4G08850.1	BP	maker-scaffold_34-snap-gene-12.110-mR	6.56
Artificial	WGBS	GO:0042330	taxis	0.03729344	0.03729344	0.51187875	AT4G08850.1	BP	maker-scaffold_34-snap-gene-12.110-mR	6.56
Artificial	WGBS	GO:0050918	positive chemotaxis	0.03729344	0.03729344	0.51187875	AT4G08850.1	BP	maker-scaffold_34-snap-gene-12.110-mR	6.56
Artificial	WGBS	GO:0080022	primary root development	0.03729344	0.03729344	0.51187875	AT4G09510.4	BP	maker-scaffold_30-augustus-gene-11.76-	6.56
Artificial	WGBS	GO:0010109	regulation of photosynthesis	0.03881935	0.03881935	0.51187875	AT5G52520.1	BP	maker-scaffold_43-augustus-gene-8.64-m	6.4173913
Artificial	WGBS	GO:0009615	response to virus	0.03968809	0.03968809	0.51187875	AT4G20910.1	BP	maker-scaffold_22-snap-gene-28.128-mR	3.98918919
Artificial	WGBS	GO:0040011	locomotion	0.04353647	0.04353647	0.51187875	AT4G08850.1	BP	maker-scaffold_34-snap-gene-12.110-mR	6.0244898
Artificial	WGBS	GO:0042325	regulation of phosphorylation	0.04522326	0.04522326	0.51187875	AT2G25760.1	BP	maker-scaffold_23-augustus-gene-10.33-	3.78461539
Artificial	WGBS	GO:0034655	nucleobase-containing compound catabo	0.04790392	0.04790392	0.51187875	AT2G13540.1	BP	maker-scaffold_7-snap-gene-23.112-mRN	2.93731343
Artificial	WGBS	GO:0008143	poly(A) binding	0.00549125	0.00549125	0.3368651	AT4G27000.1	MF	augustus_masked-scaffold_10-processed-	17.8512397
Artificial	WGBS	GO:0070717	poly-purine tract binding	0.00615116	0.00615116	0.3368651	AT4G27000.1	MF	augustus_masked-scaffold_10-processed-	16.8595041
Artificial	WGBS	GO:0010427	abscisic acid binding	0.00684535	0.00684535	0.3368651	AT1G52920.1	MF	maker-scaffold_10-snap-gene-38.56-mRN	15.9721618
Artificial	WGBS	GO:0140359	ABC-type transporter activity	0.0070749	0.0070749	0.3368651	AT5G39040.1	MF	maker-scaffold_19-augustus-gene-23.38-	5.27775782
Artificial	WGBS	GO:0008559	ABC-type xenobiotic transporter activity	0.00833472	0.00833472	0.3368651	AT1G04120.1	MF	maker-scaffold_34-snap-gene-11.72-mRN	14.4510035
Artificial	WGBS	GO:0005385	zinc ion transmembrane transporter acti	0.01081422	0.01081422	0.3368651	AT3G08650.2	MF	maker-scaffold_45-snap-gene-1.87-mRNA	12.6446281
Artificial	WGBS	GO:0019840	isoprenoid binding	0.01081422	0.01081422	0.3368651	AT1G52920.1	MF	maker-scaffold_10-snap-gene-38.56-mRN	12.6446281
Artificial	WGBS	GO:0003723	RNA binding	0.01163716	0.01163716	0.3368651	AT4G20910.1	MF	maker-scaffold_22-snap-gene-28.128-mR	1.71452584
Artificial	WGBS	GO:0033293	monocarboxylic acid binding	0.01641125	0.01641125	0.42227773	AT1G52920.1	MF	maker-scaffold_10-snap-gene-38.56-mRN	5.61983471
Artificial	WGBS	GO:0000217	DNA secondary structure binding	0.02345165	0.02345165	0.45614298	AT4G17800.1	MF	augustus_masked-scaffold_6-processed-g	8.42975207
Artificial	WGBS	GO:0043178	alcohol binding	0.02468647	0.02468647	0.45614298	AT1G52920.1	MF	maker-scaffold_10-snap-gene-38.56-mRN	8.20192093
Artificial	WGBS	GO:0045182	translation regulator activity	0.03299639	0.03299639	0.45614298	AT1G56350.1	MF	maker-scaffold_44-snap-gene-8.99-mRNA	3.31662376
Artificial	WGBS	GO:0042562	hormone binding	0.03403821	0.03403821	0.45614298	AT1G52920.1	MF	maker-scaffold_10-snap-gene-38.56-mRN	6.89706987
Artificial	WGBS	GO:0016874	ligase activity	0.04254461	0.04254461	0.45614298	AT1G08520.1	MF	maker-scaffold_7-augustus-gene-24.81-m	2.6251823
Artificial	WGBS	GO:0003729	mRNA binding	0.04442477	0.04442477	0.45614298	AT2G13540.1	MF	maker-scaffold_7-snap-gene-23.112-mRN	1.73247045
Artificial	WGBS	GO:0031406	carboxylic acid binding	0.04876023	0.04876023	0.45614298	AT1G52920.1	MF	maker-scaffold_10-snap-gene-38.56-mRN	3.67102106
Artificial	WGBS	GO:0043177	organic acid binding	0.04972727	0.04972727	0.45614298	AT1G52920.1	MF	maker-scaffold_10-snap-gene-38.56-mRN	3.64165289
Artificial	WGBS	GO:0036464	cytoplasmic ribonucleoprotein granule	0.00425471	0.00425471	0.29052388	AT1G80780.1	CC	maker-scaffold_6-snap-gene-0.78-mRNA-	6.10023791
Artificial	WGBS	GO:0035770	ribonucleoprotein granule	0.00441291	0.00441291	0.29052388	AT1G80780.1	CC	maker-scaffold_6-snap-gene-0.78-mRNA-	6.03799058
Artificial	WGBS	GO:0022627	cytosolic small ribosomal subunit	0.00563261	0.00563261	0.29052388	AT4G39200.1	CC	maker-scaffold_7-snap-gene-45.149-mRN	5.63545788
Artificial	WGBS	GO:0010494	cytoplasmic stress granule	0.01135533	0.01135533	0.31150047	AT5G21160.2	CC	maker-scaffold_32-snap-gene-15.68-mRN	12.3275641
Artificial	WGBS	GO:0015935	small ribosomal subunit	0.01207859	0.01207859	0.31150047	AT4G39200.1	CC	maker-scaffold_7-snap-gene-45.149-mRN	4.51697005
Artificial	WGBS	GO:0120025	plasma membrane bounded cell projecti	0.01207859	0.01207859	0.31150047	AT2G29560.1	CC	maker-scaffold_7-snap-gene-21.113-mRN	4.51697005
Artificial	WGBS	GO:0000932	P-body	0.01814139	0.01814139	0.35443305	AT1G80780.1	CC	maker-scaffold_6-snap-gene-0.78-mRNA-	5.41210131
Artificial	WGBS	GO:0005774	vacuolar membrane	0.02082377	0.02082377	0.35443305	AT1G80510.1	CC	augustus_masked-scaffold_32-processed-	2.01890628
Artificial	WGBS	GO:0044391	ribosomal subunit	0.02289881	0.02289881	0.35443305	AT1G43170.1	CC	augustus_masked-scaffold_1-processed-g	2.73945869
Artificial	WGBS	GO:0042995	cell projection	0.02290554	0.02290554	0.35443305	AT2G29560.1	CC	maker-scaffold_7-snap-gene-21.113-mRN	3.72152879
Artificial	WGBS	GO:0022626	cytosolic ribosome	0.02542136	0.02542136	0.35760186	AT1G43170.1	CC	augustus_masked-scaffold_1-processed-g	2.67344764
Artificial	WGBS	GO:0090406	pollen tube	0.03436558	0.03436558	0.44313512	AT4G08850.1	CC	maker-scaffold_34-snap-gene-12.110-mR	4.22659341
Artificial	WGBS	GO:1990904	ribonucleoprotein complex	0.04845044	0.04845044	0.57669752	AT1G43170.1	CC	augustus_masked-scaffold_1-processed-g	1.89925381

stress	technique	ID	Description	pvalue	p.adjust	qvalue	geneID	bgsource	PN_annoated_genes	foldEnrichme
Artificial	epiGBS	GO:0010227	floral organ abscission	0.04734979	0.04734979	0.52411376	AT3G60830..	BP	maker-scaffold_7-augustus-gene-27.78-m	2.86810785
Artificial	epiGBS	GO:0019344	cysteine biosynthetic process	0.04734979	0.04734979	0.52411376	AT4G35640..	BP	snap_masked-scaffold_18-processed-gen	2.86810785
Artificial	epiGBS	GO:0030490	maturation of SSU-rRNA	0.04741567	0.04741567	0.52411376	AT3G56510..	BP	maker-scaffold_10-augustus-gene-1.42-m	2.09893347
Artificial	epiGBS	GO:0016441	posttranscriptional gene silencing	0.04868522	0.04868522	0.52411376	AT1G24706..	BP	maker-scaffold_24-snap-gene-23.45-mRN	1.70602967
Artificial	epiGBS	GO:0044089	positive regulation of cellular componen	0.04894157	0.04894157	0.52411376	AT1G53750..	BP	maker-scaffold_8-augustus-gene-24.106-	1.96914867
Artificial	epiGBS	GO:0000966	RNA 5'-end processing	0.04898588	0.04898588	0.52411376	AT3G56510..	BP	maker-scaffold_10-augustus-gene-1.42-m	3.5339186
Artificial	epiGBS	GO:0006303	double-strand break repair via nonhomo	0.04898588	0.04898588	0.52411376	AT3G48190..	BP	maker-scaffold_19-augustus-gene-14.52-	3.5339186
Artificial	epiGBS	GO:0016925	protein sumoylation	0.04898588	0.04898588	0.52411376	AT5G60410..	BP	maker-scaffold_11-augustus-gene-12.68-	3.5339186
Artificial	epiGBS	GO:0030050	vesicle transport along actin filament	0.04898588	0.04898588	0.52411376	AT4G33200..	BP	maker-scaffold_8-snap-gene-22.92-mRNA	3.5339186
Artificial	epiGBS	GO:0032365	intracellular lipid transport	0.04898588	0.04898588	0.52411376	AT4G39850..	BP	maker-scaffold_14-augustus-gene-19.66-	3.5339186
Artificial	epiGBS	GO:0034471	ncRNA 5'-end processing	0.04898588	0.04898588	0.52411376	AT3G56510..	BP	maker-scaffold_10-augustus-gene-1.42-m	3.5339186
Artificial	epiGBS	GO:0048363	mucilage pectin metabolic process	0.04898588	0.04898588	0.52411376	AT1G18580..	BP	augustus_masked-scaffold_19-processed-	3.5339186
Artificial	epiGBS	GO:0140098	catalytic activity, acting on RNA	0.00031391	0.00031391	0.13646027	AT1G55870..	MF	maker-scaffold_10-augustus-gene-17.55-	1.71011997
Artificial	epiGBS	GO:0031267	small GTPase binding	0.00041418	0.00041418	0.13646027	AT5G62600..	MF	maker-scaffold_21-augustus-gene-4.64-m	3.83921933
Artificial	epiGBS	GO:0032182	ubiquitin-like protein binding	0.00090733	0.00090733	0.16835544	AT4G25230..	MF	maker-scaffold_6-snap-gene-32.122-mRN	2.84386617
Artificial	epiGBS	GO:0043138	3'-5' DNA helicase activity	0.00125436	0.00125436	0.16835544	AT5G61140..	MF	maker-scaffold_19-snap-gene-25.62-mRN	5.68773234
Artificial	epiGBS	GO:0051020	GTPase binding	0.00142791	0.00142791	0.16835544	AT5G62600..	MF	maker-scaffold_21-augustus-gene-4.64-m	3.26742071
Artificial	epiGBS	GO:0140223	general transcription initiation factor act	0.00215828	0.00215828	0.16835544	AT1G54140..	MF	maker-scaffold_6-augustus-gene-52.52-m	4.26579926
Artificial	epiGBS	GO:0044877	protein-containing complex binding	0.00216418	0.00216418	0.16835544	AT1G50030..	MF	maker-scaffold_9-snap-gene-28.92-mRNA	1.80669145
Artificial	epiGBS	GO:0051015	actin filament binding	0.00231618	0.00231618	0.16835544	AT2G41740..	MF	maker-scaffold_17-snap-gene-14.101-mR	2.55947955
Artificial	epiGBS	GO:0003779	actin binding	0.00247003	0.00247003	0.16835544	AT2G41740..	MF	maker-scaffold_17-snap-gene-14.101-mR	2.34200744
Artificial	epiGBS	GO:0048027	mRNA 5'-UTR binding	0.00277614	0.00277614	0.16835544	AT3G02830..	MF	maker-scaffold_24-snap-gene-25.43-mRN	6.20479892
Artificial	epiGBS	GO:0003723	RNA binding	0.00281041	0.00281041	0.16835544	AT1G53210..	MF	maker-scaffold_10-augustus-gene-21.41-	1.26286938
Artificial	epiGBS	GO:0008173	RNA methyltransferase activity	0.00495761	0.00495761	0.2404877	AT5G57280..	MF	augustus_masked-scaffold_9-processed-g	2.74229952
Artificial	epiGBS	GO:0016251	RNA polymerase II general transcription	0.00507069	0.00507069	0.2404877	AT1G54140..	MF	maker-scaffold_6-augustus-gene-52.52-m	4.26579926
Artificial	epiGBS	GO:0000339	RNA cap binding	0.00547436	0.00547436	0.2404877	AT5G44200..	MF	maker-scaffold_53-augustus-gene-3.69-m	5.25021447
Artificial	epiGBS	GO:0036002	pre-mRNA binding	0.00547436	0.00547436	0.2404877	AT1G60900..	MF	maker-scaffold_15-snap-gene-23.104-mR	5.25021447
Artificial	epiGBS	GO:0004222	metalloendopeptidase activity	0.00785588	0.00785588	0.32167375	AT3G04340..	MF	maker-scaffold_24-snap-gene-4.88-mRNA	2.55947955
Artificial	epiGBS	GO:0003724	RNA helicase activity	0.00901245	0.00901245	0.32167375	AT3G18600..	MF	maker-scaffold_9-augustus-gene-11.70-m	2.09265624
Artificial	epiGBS	GO:0008186	RNA-dependent ATPase activity	0.00901245	0.00901245	0.32167375	AT3G18600..	MF	maker-scaffold_9-augustus-gene-11.70-m	2.09265624
Artificial	epiGBS	GO:0008252	nucleotidase activity	0.00951581	0.00951581	0.32167375	AT2G23890..	MF	maker-scaffold_13-snap-gene-14.106-mR	4.55018587
Artificial	epiGBS	GO:0061659	ubiquitin-like protein ligase activity	0.00976326	0.00976326	0.32167375	AT4G25230..	MF	maker-scaffold_6-snap-gene-32.122-mRN	1.82819968
Artificial	epiGBS	GO:0008408	3'-5' exonuclease activity	0.01076419	0.01076419	0.32803161	AT1G55870..	MF	maker-scaffold_10-augustus-gene-17.55-	2.43759958
Artificial	epiGBS	GO:0004674	protein serine/threonine kinase activity	0.01095185	0.01095185	0.32803161	AT4G27290..	MF	maker-scaffold_10-augustus-gene-42.45-	1.31397721
Artificial	epiGBS	GO:0008641	ubiquitin-like modifier activating enzym	0.0121087	0.0121087	0.34691288	AT5G06460..	MF	maker-scaffold_9-snap-gene-24.107-mRN	4.26579926
Artificial	epiGBS	GO:0043130	ubiquitin binding	0.01441345	0.01441345	0.35164726	AT4G25230..	MF	maker-scaffold_6-snap-gene-32.122-mRN	2.32679959
Artificial	epiGBS	GO:0140097	catalytic activity, acting on DNA	0.01495294	0.01495294	0.35164726	AT5G55310..	MF	maker-scaffold_10-augustus-gene-13.32-	1.64644884
Artificial	epiGBS	GO:0017025	TBP-class protein binding	0.01511346	0.01511346	0.35164726	AT1G55520..	MF	maker-scaffold_24-augustus-gene-8.91-m	4.01486989
Artificial	epiGBS	GO:0019905	syntaxin binding	0.01511346	0.01511346	0.35164726	AT1G02010..	MF	maker-scaffold_3-augustus-gene-24.104-	4.01486989
Artificial	epiGBS	GO:0061630	ubiquitin protein ligase activity	0.0151254	0.0151254	0.35164726	AT4G25230..	MF	maker-scaffold_6-snap-gene-32.122-mRN	1.77959724
Artificial	epiGBS	GO:0004518	nuclease activity	0.01682192	0.01682192	0.35164726	AT1G55870..	MF	maker-scaffold_10-augustus-gene-17.55-	1.67286245
Artificial	epiGBS	GO:0008237	metallopeptidase activity	0.01729346	0.01729346	0.35164726	AT3G04340..	MF	maker-scaffold_24-snap-gene-4.88-mRNA	1.98794529
Artificial	epiGBS	GO:0003688	DNA replication origin binding	0.01765461	0.01765461	0.35164726	AT5G16690..	MF	augustus_masked-scaffold_24-processed-	5.11895911
Artificial	epiGBS	GO:0005049	nuclear export signal receptor activity	0.01765461	0.01765461	0.35164726	AT5G17020..	MF	maker-scaffold_41-augustus-gene-3.16-m	5.11895911
Artificial	epiGBS	GO:0019787	ubiquitin-like protein transferase activity	0.01837547	0.01837547	0.35164726	AT5G06460..	MF	maker-scaffold_9-snap-gene-24.107-mRN	1.44603365
Artificial	epiGBS	GO:0015385	sodium:proton antiporter activity	0.01854887	0.01854887	0.35164726	AT3G05030..	MF	augustus_masked-scaffold_28-processed-	3.79182156
Artificial	epiGBS	GO:0051139	metal ion:proton antiporter activity	0.01881281	0.01881281	0.35164726	AT1G53210..	MF	maker-scaffold_10-augustus-gene-21.41-	3.1598513
Artificial	epiGBS	GO:0016877	ligase activity, forming carbon-sulfur bo	0.0192114	0.0192114	0.35164726	AT5G06460..	MF	maker-scaffold_9-snap-gene-24.107-mRN	2.35354442
Artificial	epiGBS	GO:0140142	nucleocytoplasmic carrier activity	0.02182765	0.02182765	0.36049916	AT5G17020..	MF	maker-scaffold_41-augustus-gene-3.16-m	3.04699947
Artificial	epiGBS	GO:0009678	pyrophosphate hydrolysis-driven proton	0.02206609	0.02206609	0.36049916	AT4G11150..	MF	maker-scaffold_24-augustus-gene-5.51-m	2.694189

stress	technique	ID	Description	pvalue	p.adjust	qvalue	geneID	bgsource	PN_annoated_genes	foldEnrichme
Artificial	epiGBS	GO:0010638	positive regulation of organelle organiza	0.00566826	0.00566826	0.24841675	AT3G52140.	BP	maker-scaffold_7-augustus-gene-29.88-m	2.21021713
Artificial	epiGBS	GO:0006367	transcription initiation from RNA polyme	0.0059259	0.0059259	0.25160048	AT1G54140.	BP	maker-scaffold_6-augustus-gene-52.52-m	2.86810785
Artificial	epiGBS	GO:0032956	regulation of actin cytoskeleton organiza	0.00617401	0.00617401	0.25160048	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	2.65043895
Artificial	epiGBS	GO:0035725	sodium ion transmembrane transport	0.00618009	0.00618009	0.25160048	AT1G53210.	BP	maker-scaffold_10-augustus-gene-21.41-	5.07434465
Artificial	epiGBS	GO:0007015	actin filament organization	0.00618252	0.00618252	0.25160048	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	2.18766389
Artificial	epiGBS	GO:0022607	cellular component assembly	0.00669691	0.00669691	0.26775285	AT3G56510.	BP	maker-scaffold_10-augustus-gene-1.42-m	1.31999796
Artificial	epiGBS	GO:0032970	regulation of actin filament-based proce	0.00694702	0.00694702	0.27217935	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	2.60394002
Artificial	epiGBS	GO:0010150	leaf senescence	0.00705812	0.00705812	0.27217935	AT5G22070.	BP	augustus_masked-scaffold_7-processed-g	1.76124098
Artificial	epiGBS	GO:0033044	regulation of chromosome organization	0.00719206	0.00719206	0.27217935	AT3G48190.	BP	maker-scaffold_19-augustus-gene-14.52-	2.3257413
Artificial	epiGBS	GO:0044085	cellular component biogenesis	0.00728554	0.00728554	0.27217935	AT5G14580.	BP	snap_masked-scaffold_10-processed-gen	1.23943232
Artificial	epiGBS	GO:0065003	protein-containing complex assembly	0.00748996	0.00748996	0.27217935	AT3G56510.	BP	maker-scaffold_10-augustus-gene-1.42-m	1.39759493
Artificial	epiGBS	GO:0043414	macromolecule methylation	0.00768765	0.00768765	0.27217935	AT5G57280.	BP	augustus_masked-scaffold_9-processed-g	1.66194621
Artificial	epiGBS	GO:0034599	cellular response to oxidative stress	0.00801354	0.00801354	0.27217935	AT5G26860.	BP	maker-scaffold_44-augustus-gene-8.88-m	2.19888268
Artificial	epiGBS	GO:0010216	maintenance of DNA methylation	0.00824203	0.00824203	0.27217935	AT5G20320.	BP	maker-scaffold_8-snap-gene-37.93-mRNA	4.71189146
Artificial	epiGBS	GO:0140029	exocytic process	0.00824203	0.00824203	0.27217935	AT1G02010.	BP	maker-scaffold_3-augustus-gene-24.104-	4.71189146
Artificial	epiGBS	GO:0006289	nucleotide-excision repair	0.00824845	0.00824845	0.27217935	AT1G05055.	BP	maker-scaffold_42-augustus-gene-1.44-m	2.96003438
Artificial	epiGBS	GO:0030041	actin filament polymerization	0.00824845	0.00824845	0.27217935	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	2.96003438
Artificial	epiGBS	GO:0030833	regulation of actin filament polymerizat	0.00824845	0.00824845	0.27217935	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	2.96003438
Artificial	epiGBS	GO:0009991	response to extracellular stimulus	0.0083988	0.0083988	0.27217935	AT5G60410.	BP	maker-scaffold_11-augustus-gene-12.68-	1.56133682
Artificial	epiGBS	GO:0043933	protein-containing complex subunit orga	0.00847968	0.00847968	0.27217935	AT3G56510.	BP	maker-scaffold_10-augustus-gene-1.42-m	1.36312849
Artificial	epiGBS	GO:0090693	plant organ senescence	0.00877852	0.00877852	0.27631809	AT5G22070.	BP	augustus_masked-scaffold_7-processed-g	1.72769354
Artificial	epiGBS	GO:0031398	positive regulation of protein ubiquitinat	0.00899777	0.00899777	0.27631809	AT4G22910.	BP	maker-scaffold_6-snap-gene-26.99-mRNA	3.74809548
Artificial	epiGBS	GO:1903322	positive regulation of protein modificati	0.00899777	0.00899777	0.27631809	AT4G22910.	BP	maker-scaffold_6-snap-gene-26.99-mRNA	3.74809548
Artificial	epiGBS	GO:0034660	ncRNA metabolic process	0.00931612	0.00931612	0.27631809	AT5G14580.	BP	snap_masked-scaffold_10-processed-gen	1.44260693
Artificial	epiGBS	GO:0006914	autophagy	0.00933611	0.00933611	0.27631809	AT1G50030.	BP	maker-scaffold_9-snap-gene-28.92-mRNA	2.08146662
Artificial	epiGBS	GO:0061919	process utilizing autophagic mechanism	0.00933611	0.00933611	0.27631809	AT1G50030.	BP	maker-scaffold_9-snap-gene-28.92-mRNA	2.08146662
Artificial	epiGBS	GO:0010192	mucilage biosynthetic process	0.00948745	0.00948745	0.2771973	AT1G18580.	BP	augustus_masked-scaffold_19-processed-	2.88603352
Artificial	epiGBS	GO:0006325	chromatin organization	0.01038501	0.01038501	0.29665574	AT5G55310.	BP	maker-scaffold_10-augustus-gene-13.32-	1.47802256
Artificial	epiGBS	GO:0051443	positive regulation of ubiquitin-protein t	0.01070766	0.01070766	0.29665574	AT4G22910.	BP	maker-scaffold_6-snap-gene-26.99-mRNA	4.39776536
Artificial	epiGBS	GO:0035195	gene silencing by miRNA	0.01079984	0.01079984	0.29665574	AT1G24706.	BP	maker-scaffold_24-snap-gene-23.45-mRN	2.43318985
Artificial	epiGBS	GO:0035196	production of miRNAs involved in gene s	0.01085708	0.01085708	0.29665574	AT1G24706.	BP	maker-scaffold_24-snap-gene-23.45-mRN	2.81564246
Artificial	epiGBS	GO:0030838	positive regulation of actin filament poly	0.01093447	0.01093447	0.29665574	AT2G30910.	BP	maker-scaffold_3-augustus-gene-50.123-	3.58513481
Artificial	epiGBS	GO:0045010	actin nucleation	0.01093447	0.01093447	0.29665574	AT2G30910.	BP	maker-scaffold_3-augustus-gene-50.123-	3.58513481
Artificial	epiGBS	GO:0090305	nucleic acid phosphodiester bond hydrol	0.01110553	0.01110553	0.29775199	AT1G55870.	BP	maker-scaffold_10-augustus-gene-17.55-	1.64916201
Artificial	epiGBS	GO:0034470	ncRNA processing	0.01158096	0.01158096	0.30688842	AT5G14580.	BP	snap_masked-scaffold_10-processed-gen	1.43702914
Artificial	epiGBS	GO:0097435	supramolecular fiber organization	0.01178077	0.01178077	0.30859496	AT3G27120.	BP	maker-scaffold_9-augustus-gene-47.67-m	1.76034147
Artificial	epiGBS	GO:0008154	actin polymerization or depolymerizatio	0.01242233	0.01242233	0.31896069	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	2.53717233
Artificial	epiGBS	GO:0051247	positive regulation of protein metabolic	0.01245641	0.01245641	0.31896069	AT4G22910.	BP	maker-scaffold_6-snap-gene-26.99-mRNA	1.75050716
Artificial	epiGBS	GO:0044272	sulfur compound biosynthetic process	0.01273114	0.01273114	0.32237331	AT2G43360.	BP	maker-scaffold_14-augustus-gene-31.94-	1.77753989
Artificial	epiGBS	GO:0051493	regulation of cytoskeleton organization	0.01314276	0.01314276	0.32841577	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	2.06145251
Artificial	epiGBS	GO:0006406	mRNA export from nucleus	0.01325799	0.01325799	0.32841577	AT5G16790.	BP	maker-scaffold_24-augustus-gene-20.60-	2.99847638
Artificial	epiGBS	GO:0071805	potassium ion transmembrane transport	0.01353108	0.01353108	0.33157657	AT2G26650.	BP	maker-scaffold_4-augustus-gene-4.36-mR	2.22859731
Artificial	epiGBS	GO:0000904	cell morphogenesis involved in different	0.01398005	0.01398005	0.33625805	AT4G22910.	BP	maker-scaffold_6-snap-gene-26.99-mRNA	1.58222186
Artificial	epiGBS	GO:0006405	RNA export from nucleus	0.01401722	0.01401722	0.33625805	AT5G16790.	BP	maker-scaffold_24-augustus-gene-20.60-	2.68468234
Artificial	epiGBS	GO:0007010	cytoskeleton organization	0.01431098	0.01431098	0.33709125	AT3G27120.	BP	maker-scaffold_9-augustus-gene-47.67-m	1.51254504
Artificial	epiGBS	GO:0071103	DNA conformation change	0.01456041	0.01456041	0.33709125	AT5G55310.	BP	maker-scaffold_10-augustus-gene-13.32-	1.78571683
Artificial	epiGBS	GO:0031401	positive regulation of protein modificati	0.0146149	0.0146149	0.33709125	AT4G22910.	BP	maker-scaffold_6-snap-gene-26.99-mRNA	2.10939327
Artificial	epiGBS	GO:0048519	negative regulation of biological process	0.0147313	0.0147313	0.33709125	AT5G14580.	BP	snap_masked-scaffold_10-processed-gen	1.24367284
Artificial	epiGBS	GO:0001510	RNA methylation	0.01479153	0.01479153	0.33709125	AT5G57280.	BP	augustus_masked-scaffold_9-processed-g	2.19888268
Artificial	epiGBS	GO:0051241	negative regulation of multicellular orga	0.01542691	0.01542691	0.34809023	AT1G30970.	BP	snap_masked-scaffold_6-processed-gene	1.77441482

stress	technique	ID	Description	pvalue	p.adjust	qvalue	geneID	bgsource	PN_annoated_genes	foldEnrichme
Artificial	epiGBS	GO:0005765	lysosomal membrane	0.00672642	0.00672642	0.13610173	AT5G64500.	CC	maker-scaffold_7-augustus-gene-34.102-	4.00595759
Artificial	epiGBS	GO:0098852	lytic vacuole membrane	0.00672642	0.00672642	0.13610173	AT5G64500.	CC	maker-scaffold_7-augustus-gene-34.102-	4.00595759
Artificial	epiGBS	GO:0005730	nucleolus	0.00789579	0.00789579	0.15135417	AT5G55310.	CC	maker-scaffold_10-augustus-gene-13.32-	1.43191676
Artificial	epiGBS	GO:0016592	mediator complex	0.00879244	0.00879244	0.16011503	AT1G44910.	CC	maker-scaffold_9-snap-gene-28.97-mRNA	2.6920035
Artificial	epiGBS	GO:0005686	U2 snRNP	0.01007948	0.01007948	0.17481205	AT5G12190.	CC	augustus_masked-scaffold_9-processed-g	3.65761345
Artificial	epiGBS	GO:0090575	RNA polymerase II transcription regulato	0.01189544	0.01189544	0.19692935	AT1G54140.	CC	maker-scaffold_6-augustus-gene-52.52-m	2.2736516
Artificial	epiGBS	GO:0005856	cytoskeleton	0.01291183	0.01291183	0.20084834	AT3G60830.	CC	maker-scaffold_7-augustus-gene-27.78-m	1.42136727
Artificial	epiGBS	GO:0000151	ubiquitin ligase complex	0.01378795	0.01378795	0.20084834	AT4G22910.	CC	maker-scaffold_6-snap-gene-26.99-mRNA	1.52954744
Artificial	epiGBS	GO:0015629	actin cytoskeleton	0.01421583	0.01421583	0.20084834	AT3G60830.	CC	maker-scaffold_7-augustus-gene-27.78-m	2.21381867
Artificial	epiGBS	GO:0016604	nuclear body	0.01433802	0.01433802	0.20084834	AT1G72390.	CC	maker-scaffold_6-snap-gene-51.77-mRNA	1.86944688
Artificial	epiGBS	GO:0005675	transcription factor TFIIH holo complex	0.01833515	0.01833515	0.24053618	AT1G05055.	CC	maker-scaffold_42-augustus-gene-1.44-m	5.04750656
Artificial	epiGBS	GO:0010369	chromocenter	0.01944105	0.01944105	0.24053618	AT3G25980.	CC	maker-scaffold_25-augustus-gene-13.86-	3.73889375
Artificial	epiGBS	GO:0080008	Cul4-RING E3 ubiquitin ligase complex	0.01987713	0.01987713	0.24053618	AT1G49040.	CC	maker-scaffold_31-snap-gene-2.49-mRNA	1.75947941
Artificial	epiGBS	GO:0030684	preribosome	0.02035675	0.02035675	0.24053618	AT5G16750.	CC	augustus_masked-scaffold_24-processed-	2.0116874
Artificial	epiGBS	GO:0019898	extrinsic component of membrane	0.02047338	0.02047338	0.24053618	AT2G28150.	CC	maker-scaffold_7-snap-gene-2.85-mRNA-	1.94134868
Artificial	epiGBS	GO:0005764	lysosome	0.02728011	0.02728011	0.30366338	AT1G47128.	CC	maker-scaffold_9-snap-gene-33.131-mRN	2.20656025
Artificial	epiGBS	GO:0005669	transcription factor TFIID complex	0.02802126	0.02802126	0.30366338	AT1G54140.	CC	maker-scaffold_6-augustus-gene-52.52-m	3.36500437
Artificial	epiGBS	GO:0005773	vacuole	0.02834777	0.02834777	0.30366338	AT1G53210.	CC	maker-scaffold_10-augustus-gene-21.41-	1.20523079
Artificial	epiGBS	GO:0030008	TRAPP complex	0.03075934	0.03075934	0.31875194	AT5G54750.	CC	maker-scaffold_10-augustus-gene-47.66-	4.20625547
Artificial	epiGBS	GO:0097525	spliceosomal snRNP complex	0.03237077	0.03237077	0.31875194	AT1G44910.	CC	maker-scaffold_9-snap-gene-28.97-mRNA	2.13651071
Artificial	epiGBS	GO:0005684	U2-type spliceosomal complex	0.03325707	0.03325707	0.31875194	AT1G44910.	CC	maker-scaffold_9-snap-gene-28.97-mRNA	2.26490679
Artificial	epiGBS	GO:0031965	nuclear membrane	0.03325707	0.03325707	0.31875194	AT4G33200.	CC	maker-scaffold_8-snap-gene-22.92-mRNA	2.26490679
Artificial	epiGBS	GO:0030532	small nuclear ribonucleoprotein complex	0.03514383	0.03514383	0.32819883	AT1G44910.	CC	maker-scaffold_9-snap-gene-28.97-mRNA	2.10312773
Artificial	epiGBS	GO:0005819	spindle	0.03683626	0.03683626	0.33177913	AT3G57890.	CC	maker-scaffold_17-augustus-gene-11.87-	1.73591496
Artificial	epiGBS	GO:0005849	mRNA cleavage factor complex	0.03826008	0.03826008	0.33177913	AT3G04680.	CC	maker-scaffold_27-snap-gene-6.52-mRNA	3.88269736
Artificial	epiGBS	GO:0005885	Arp2/3 protein complex	0.03826008	0.03826008	0.33177913	AT2G30910.	CC	maker-scaffold_3-augustus-gene-50.123-	3.88269736
Artificial	epiGBS	GO:0055029	nuclear DNA-directed RNA polymerase c	0.03972506	0.03972506	0.33277956	AT5G60040.	CC	maker-scaffold_10-snap-gene-1.54-mRNA	1.81446314
Artificial	epiGBS	GO:0005694	chromosome	0.04112043	0.04112043	0.33277956	AT5G55310.	CC	maker-scaffold_10-augustus-gene-13.32-	1.38716936
Artificial	epiGBS	GO:0030133	transport vesicle	0.04134676	0.04134676	0.33277956	AT5G13300.	CC	maker-scaffold_6-augustus-gene-7.62-mR	1.94134868
Artificial	epiGBS	GO:0015630	microtubule cytoskeleton	0.04411031	0.04411031	0.33277956	AT3G60830.	CC	maker-scaffold_7-augustus-gene-27.78-m	1.38667763
Artificial	epiGBS	GO:0000323	lytic vacuole	0.04441163	0.04441163	0.33277956	AT1G47128.	CC	maker-scaffold_9-snap-gene-33.131-mRN	2.00895784
Artificial	epiGBS	GO:0016591	RNA polymerase II, holoenzyme	0.04441163	0.04441163	0.33277956	AT1G54140.	CC	maker-scaffold_6-augustus-gene-52.52-m	2.00895784
Artificial	epiGBS	GO:0031982	vesicle	0.0456536	0.0456536	0.33277956	AT1G47128.	CC	maker-scaffold_9-snap-gene-33.131-mRN	1.21451072
Artificial	epiGBS	GO:0032806	carboxy-terminal domain protein kinase	0.04659876	0.04659876	0.33277956	AT1G05055.	CC	maker-scaffold_42-augustus-gene-1.44-m	3.60536183
Artificial	epiGBS	GO:0035097	histone methyltransferase complex	0.04659876	0.04659876	0.33277956	AT2G19520.	CC	augustus_masked-scaffold_13-processed-	3.60536183

stress	technique	ID	Description	pvalue	p.adjust	qvalue	geneID	bgsource	PN_annoated_genes	foldEnrichme
Artificial	epiGBS	GO:0007568	aging	0.01590054	0.01590054	0.3552598	AT2G28800.	BP	maker-scaffold_9-snap-gene-6.79-mRNA-	1.56489826
Artificial	epiGBS	GO:0051258	protein polymerization	0.01757207	0.01757207	0.38879428	AT2G28150.	BP	maker-scaffold_7-snap-gene-2.85-mRNA-	2.14176885
Artificial	epiGBS	GO:0031334	positive regulation of protein-containing	0.01778519	0.01778519	0.389726	AT1G53750.	BP	maker-scaffold_8-augustus-gene-24.106-	2.56536313
Artificial	epiGBS	GO:0048468	cell development	0.01799506	0.01799506	0.39009137	AT4G22910.	BP	maker-scaffold_6-snap-gene-26.99-mRNA	1.45640282
Artificial	epiGBS	GO:0010608	posttranscriptional regulation of gene ex	0.01865021	0.01865021	0.39009137	AT1G24706.	BP	maker-scaffold_24-snap-gene-23.45-mRN	1.50395278
Artificial	epiGBS	GO:0042180	cellular ketone metabolic process	0.01871679	0.01871679	0.39009137	AT1G50030.	BP	maker-scaffold_9-snap-gene-28.92-mRNA	1.70602967
Artificial	epiGBS	GO:0031667	response to nutrient levels	0.01878133	0.01878133	0.39009137	AT5G60410.	BP	maker-scaffold_11-augustus-gene-12.68-	1.54238174
Artificial	epiGBS	GO:0080135	regulation of cellular response to stress	0.01907334	0.01907334	0.39009137	AT4G25230.	BP	maker-scaffold_6-snap-gene-32.122-mRN	1.95940041
Artificial	epiGBS	GO:1900140	regulation of seedling development	0.01907334	0.01907334	0.39009137	AT4G39850.	BP	maker-scaffold_14-augustus-gene-19.66-	1.95940041
Artificial	epiGBS	GO:0030036	actin cytoskeleton organization	0.01929268	0.01929268	0.39009137	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	1.89726603
Artificial	epiGBS	GO:0032456	endocytic recycling	0.01934241	0.01934241	0.39009137	AT2G27900.	BP	maker-scaffold_7-snap-gene-1.74-mRNA-	4.94748603
Artificial	epiGBS	GO:2000779	regulation of double-strand break repair	0.01934241	0.01934241	0.39009137	AT3G48710.	BP	maker-scaffold_19-augustus-gene-5.104-	4.94748603
Artificial	epiGBS	GO:0030004	cellular monovalent inorganic cation hor	0.01991378	0.01991378	0.39809165	AT5G62670.	BP	maker-scaffold_21-augustus-gene-3.67-m	2.50959437
Artificial	epiGBS	GO:0018105	peptidyl-serine phosphorylation	0.02061142	0.02061142	0.4044438	AT3G59410.	BP	maker-scaffold_14-augustus-gene-32.79-	1.88062335
Artificial	epiGBS	GO:0071824	protein-DNA complex subunit organizati	0.02061142	0.02061142	0.4044438	AT1G54140.	BP	maker-scaffold_6-augustus-gene-52.52-m	1.88062335
Artificial	epiGBS	GO:0000380	alternative mRNA splicing, via spliceoso	0.02077186	0.02077186	0.4044438	AT2G27100.	BP	maker-scaffold_16-augustus-gene-12.88-	3.66480447
Artificial	epiGBS	GO:1902903	regulation of supramolecular fiber orgar	0.02094141	0.02094141	0.4044438	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	2.31461335
Artificial	epiGBS	GO:0009892	negative regulation of metabolic process	0.02127535	0.02127535	0.40744039	AT5G14580.	BP	snap_masked-scaffold_10-processed-gen	1.30604777
Artificial	epiGBS	GO:2000242	negative regulation of reproductive proc	0.02154586	0.02154586	0.40918225	AT3G27120.	BP	maker-scaffold_9-augustus-gene-47.67-m	1.99349254
Artificial	epiGBS	GO:0018209	peptidyl-serine modification	0.02199645	0.02199645	0.41428714	AT3G59410.	BP	maker-scaffold_14-augustus-gene-32.79-	1.8642701
Artificial	epiGBS	GO:0048366	leaf development	0.02226553	0.02226553	0.41591785	AT5G55540.	BP	maker-scaffold_10-augustus-gene-14.12-	1.38273298
Artificial	epiGBS	GO:0031047	gene silencing by RNA	0.02266408	0.02266408	0.4199207	AT1G24706.	BP	maker-scaffold_24-snap-gene-23.45-mRN	1.69913662
Artificial	epiGBS	GO:0051607	defense response to virus	0.02426007	0.02426007	0.44304757	AT1G50030.	BP	maker-scaffold_9-snap-gene-28.92-mRNA	2.03600248
Artificial	epiGBS	GO:0010191	mucilage metabolic process	0.02469117	0.02469117	0.44304757	AT1G18580.	BP	augustus_masked-scaffold_19-processed-	2.40502793
Artificial	epiGBS	GO:0051347	positive regulation of transferase activit	0.02469117	0.02469117	0.44304757	AT4G22910.	BP	maker-scaffold_6-snap-gene-26.99-mRNA	2.40502793
Artificial	epiGBS	GO:0030705	cytoskeleton-dependent intracellular tra	0.02488532	0.02488532	0.44304757	AT4G33200.	BP	maker-scaffold_8-snap-gene-22.92-mRNA	2.94493216
Artificial	epiGBS	GO:0046496	nicotinamide nucleotide metabolic proce	0.02507874	0.02507874	0.44304757	AT3G21070.	BP	maker-scaffold_9-augustus-gene-14.82-m	3.47192002
Artificial	epiGBS	GO:0051438	regulation of ubiquitin-protein transfera	0.02507874	0.02507874	0.44304757	AT4G22910.	BP	maker-scaffold_6-snap-gene-26.99-mRNA	3.47192002
Artificial	epiGBS	GO:0090332	stomatal closure	0.0261846	0.0261846	0.45902554	AT1G47128.	BP	maker-scaffold_9-snap-gene-33.131-mRN	2.01117318
Artificial	epiGBS	GO:0016072	rRNA metabolic process	0.02676321	0.02676321	0.46277799	AT5G14580.	BP	snap_masked-scaffold_10-processed-gen	1.49401437
Artificial	epiGBS	GO:0010565	regulation of cellular ketone metabolic p	0.02680479	0.02680479	0.46277799	AT1G50030.	BP	maker-scaffold_9-snap-gene-28.92-mRNA	1.86697586
Artificial	epiGBS	GO:0030029	actin filament-based process	0.02823579	0.02823579	0.46838628	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	1.80160556
Artificial	epiGBS	GO:0006893	Golgi to plasma membrane transport	0.02861452	0.02861452	0.46838628	AT1G10180.	BP	maker-scaffold_80-augustus-gene-1.96-m	2.84338278
Artificial	epiGBS	GO:0070897	transcription preinitiation complex asser	0.02861452	0.02861452	0.46838628	AT1G54140.	BP	maker-scaffold_6-augustus-gene-52.52-m	2.84338278
Artificial	epiGBS	GO:0006338	chromatin remodeling	0.02862616	0.02862616	0.46838628	AT5G55310.	BP	maker-scaffold_10-augustus-gene-13.32-	1.90955601
Artificial	epiGBS	GO:0010029	regulation of seed germination	0.02862616	0.02862616	0.46838628	AT4G39850.	BP	maker-scaffold_14-augustus-gene-19.66-	1.90955601
Artificial	epiGBS	GO:0032270	positive regulation of cellular protein me	0.02923505	0.02923505	0.46838628	AT4G22910.	BP	maker-scaffold_6-snap-gene-26.99-mRNA	1.64916201
Artificial	epiGBS	GO:0090626	plant epidermis morphogenesis	0.02927824	0.02927824	0.46838628	AT4G22910.	BP	maker-scaffold_6-snap-gene-26.99-mRNA	1.74911122
Artificial	epiGBS	GO:0070647	protein modification by small protein co	0.02977939	0.02977939	0.46838628	AT1G47128.	BP	maker-scaffold_9-snap-gene-33.131-mRN	1.26706325
Artificial	epiGBS	GO:0051123	RNA polymerase II preinitiation complex	0.02988387	0.02988387	0.46838628	AT1G54140.	BP	maker-scaffold_6-augustus-gene-52.52-m	3.29832402
Artificial	epiGBS	GO:0060321	acceptance of pollen	0.02988387	0.02988387	0.46838628	AT1G10180.	BP	maker-scaffold_80-augustus-gene-1.96-m	3.29832402
Artificial	epiGBS	GO:0016570	histone modification	0.03019506	0.03019506	0.46838628	AT5G58575.	BP	snap_masked-scaffold_9-processed-gene	1.55302252
Artificial	epiGBS	GO:0032271	regulation of protein polymerization	0.03019974	0.03019974	0.46838628	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	2.30882682
Artificial	epiGBS	GO:0032535	regulation of cellular component size	0.0303568	0.0303568	0.46838628	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	1.96328811
Artificial	epiGBS	GO:0090066	regulation of anatomical structure size	0.0303568	0.0303568	0.46838628	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	1.96328811
Artificial	epiGBS	GO:0006403	RNA localization	0.03062989	0.03062989	0.46838628	AT5G16790.	BP	maker-scaffold_24-augustus-gene-20.60-	1.8896648
Artificial	epiGBS	GO:0010119	regulation of stomatal movement	0.03163875	0.03163875	0.46838628	AT1G47128.	BP	maker-scaffold_9-snap-gene-33.131-mRN	1.69434453
Artificial	epiGBS	GO:0007264	small GTPase mediated signal transduct	0.03215047	0.03215047	0.46838628	AT1G49040.	BP	maker-scaffold_31-snap-gene-2.49-mRNA	2.47374302
Artificial	epiGBS	GO:0006265	DNA topological change	0.0323916	0.0323916	0.46838628	AT5G55310.	BP	maker-scaffold_10-augustus-gene-13.32-	4.12290503
Artificial	epiGBS	GO:0010508	positive regulation of autophagy	0.0323916	0.0323916	0.46838628	AT3G01090.	BP	maker-scaffold_42-augustus-gene-3.26-m	4.12290503

stress	technique	ID	Description	pvalue	p.adjust	qvalue	geneID	bgsource	PN_annoated_genes	foldEnrichme
Insect	epiGBS	GO:0090503	RNA phosphodiester bond hydrolysis, exo	0.02963679	0.02963679	0.63355117	AT1G55870.	BP	maker-scaffold_10-augustus-gene-17.55-	2.53935484
Insect	epiGBS	GO:0044281	small molecule metabolic process	0.02976135	0.02976135	0.63355117	AT5G54080.	BP	maker-scaffold_10-augustus-gene-20.65-	1.18883654
Insect	epiGBS	GO:0033865	nucleoside bisphosphate metabolic proc	0.03064709	0.03064709	0.63355117	AT1G09430.	BP	maker-scaffold_44-snap-gene-9.114-mRN	2.17038875
Insect	epiGBS	GO:0033875	ribonucleoside bisphosphate metabolic p	0.03064709	0.03064709	0.63355117	AT1G09430.	BP	maker-scaffold_44-snap-gene-9.114-mRN	2.17038875
Insect	epiGBS	GO:0034032	purine nucleoside bisphosphate metabol	0.03064709	0.03064709	0.63355117	AT1G09430.	BP	maker-scaffold_44-snap-gene-9.114-mRN	2.17038875
Insect	epiGBS	GO:0010228	vegetative to reproductive phase transiti	0.03076274	0.03076274	0.63355117	AT1G72390.	BP	maker-scaffold_6-snap-gene-51.77-mRNA	1.57333014
Insect	epiGBS	GO:0010256	endomembrane system organization	0.03087731	0.03087731	0.63355117	AT2G06530.	BP	maker-scaffold_6-augustus-gene-54.60-m	1.78634269
Insect	epiGBS	GO:0051603	proteolysis involved in cellular protein ca	0.03099778	0.03099778	0.63355117	AT5G06460.	BP	maker-scaffold_9-snap-gene-24.107-mRN	1.36989614
Insect	epiGBS	GO:0006790	sulfur compound metabolic process	0.03176335	0.03176335	0.64324226	AT1G64660.	BP	augustus_masked-scaffold_6-processed-g	1.46126557
Insect	epiGBS	GO:0010030	positive regulation of seed germination	0.03232786	0.03232786	0.64872266	AT2G19450.	BP	maker-scaffold_13-snap-gene-33.63-mRN	3.25558313
Insect	epiGBS	GO:0051168	nuclear export	0.03319389	0.03319389	0.65917791	AT1G24706.	BP	maker-scaffold_24-snap-gene-23.45-mRN	2.27890819
Insect	epiGBS	GO:0120251	hydrocarbon biosynthetic process	0.03344613	0.03344613	0.65917791	AT4G14210.	BP	maker-scaffold_34-snap-gene-3.69-mRNA	1.94140278
Insect	epiGBS	GO:0000280	nuclear division	0.03414307	0.03414307	0.66695875	AT3G18524.	BP	maker-scaffold_31-augustus-gene-3.35-m	1.6516129
Insect	epiGBS	GO:0090501	RNA phosphodiester bond hydrolysis	0.03635165	0.03635165	0.68199829	AT1G55870.	BP	maker-scaffold_10-augustus-gene-17.55-	1.84741424
Insect	epiGBS	GO:0006310	DNA recombination	0.03663411	0.03663411	0.68199829	AT1G79950.	BP	maker-scaffold_6-snap-gene-59.48-mRNA	1.70263256
Insect	epiGBS	GO:0007059	chromosome segregation	0.03683451	0.03683451	0.68199829	AT5G47690.	BP	maker-scaffold_4-snap-gene-69.82-mRNA	1.78827806
Insect	epiGBS	GO:0006825	copper ion transport	0.0371634	0.0371634	0.68199829	AT4G33520.	BP	maker-scaffold_64-augustus-gene-3.76-m	3.96774194
Insect	epiGBS	GO:0016074	snoRNA metabolic process	0.0371634	0.0371634	0.68199829	AT5G52470.	BP	maker-scaffold_25-snap-gene-18.70-mRN	3.96774194
Insect	epiGBS	GO:0042304	regulation of fatty acid biosynthetic proc	0.0371634	0.0371634	0.68199829	AT4G19045.	BP	maker-scaffold_6-snap-gene-35.99-mRNA	3.96774194
Insect	epiGBS	GO:0043144	snoRNA processing	0.0371634	0.0371634	0.68199829	AT5G52470.	BP	maker-scaffold_25-snap-gene-18.70-mRN	3.96774194
Insect	epiGBS	GO:0019318	hexose metabolic process	0.03851878	0.03851878	0.68199829	AT1G76270.	BP	maker-scaffold_7-augustus-gene-52.110-	1.73016839
Insect	epiGBS	GO:0044257	cellular protein catabolic process	0.03854635	0.03854635	0.68199829	AT5G06460.	BP	maker-scaffold_9-snap-gene-24.107-mRN	1.3469498
Insect	epiGBS	GO:0030258	lipid modification	0.03855788	0.03855788	0.68199829	AT2G42790.	BP	maker-scaffold_17-augustus-gene-31.40-	1.77577261
Insect	epiGBS	GO:0043632	modification-dependent macromolecule	0.03855858	0.03855858	0.68199829	AT5G06460.	BP	maker-scaffold_9-snap-gene-24.107-mRN	1.37306082
Insect	epiGBS	GO:0009150	purine ribonucleotide metabolic process	0.03862055	0.03862055	0.68199829	AT1G09430.	BP	maker-scaffold_44-snap-gene-9.114-mRN	1.59885305
Insect	epiGBS	GO:0019752	carboxylic acid metabolic process	0.0395478	0.0395478	0.69282989	AT5G54080.	BP	maker-scaffold_10-augustus-gene-20.65-	1.23728955
Insect	epiGBS	GO:0043436	oxoacid metabolic process	0.04021085	0.04021085	0.69839099	AT5G54080.	BP	maker-scaffold_10-augustus-gene-20.65-	1.22772998
Insect	epiGBS	GO:0030163	protein catabolic process	0.04071814	0.04071814	0.69839099	AT5G06460.	BP	maker-scaffold_9-snap-gene-24.107-mRN	1.33542123
Insect	epiGBS	GO:0031503	protein-containing complex localization	0.04101213	0.04101213	0.69839099	AT1G24706.	BP	maker-scaffold_24-snap-gene-23.45-mRN	2.17836812
Insect	epiGBS	GO:0043628	ncRNA 3'-end processing	0.0411308	0.0411308	0.69839099	AT3G60500.	BP	maker-scaffold_7-snap-gene-23.104-mRN	3.02304148
Insect	epiGBS	GO:0006259	DNA metabolic process	0.04254017	0.04254017	0.70095791	AT1G79950.	BP	maker-scaffold_6-snap-gene-59.48-mRNA	1.37634409
Insect	epiGBS	GO:0032259	methylation	0.04271117	0.04271117	0.70095791	AT3G27180.	BP	maker-scaffold_9-snap-gene-48.74-mRNA	1.39219015
Insect	epiGBS	GO:0016192	vesicle-mediated transport	0.04350719	0.04350719	0.70095791	AT2G06530.	BP	maker-scaffold_6-augustus-gene-54.60-m	1.34594779
Insect	epiGBS	GO:0000394	RNA splicing, via endonucleolytic cleava	0.0435852	0.0435852	0.70095791	AT1G07910.	BP	augustus_masked-scaffold_17-processed-	3.73434535
Insect	epiGBS	GO:0006144	purine nucleobase metabolic process	0.0435852	0.0435852	0.70095791	AT4G22570.	BP	maker-scaffold_6-snap-gene-30.75-mRNA	3.73434535
Insect	epiGBS	GO:0036297	interstrand cross-link repair	0.0435852	0.0435852	0.70095791	AT1G79950.	BP	maker-scaffold_6-snap-gene-59.48-mRNA	3.73434535
Insect	epiGBS	GO:0080142	regulation of salicylic acid biosynthetic p	0.0435852	0.0435852	0.70095791	AT1G53130.	BP	augustus_masked-scaffold_10-processed-	3.73434535
Insect	epiGBS	GO:0045814	negative regulation of gene expression,	0.04386252	0.04386252	0.70095791	AT1G08620.	BP	maker-scaffold_24-augustus-gene-3.78-m	2.14679757
Insect	epiGBS	GO:0051239	regulation of multicellular organismal pr	0.04433159	0.04433159	0.70095791	AT5G60910.	BP	maker-scaffold_9-snap-gene-46.62-mRNA	1.36391129
Insect	epiGBS	GO:0045132	meiotic chromosome segregation	0.04445751	0.04445751	0.70095791	AT5G05510.	BP	maker-scaffold_5-augustus-gene-45.107-	2.3085044
Insect	epiGBS	GO:0048580	regulation of post-embryonic developme	0.04587093	0.04587093	0.70563314	AT5G60910.	BP	maker-scaffold_9-snap-gene-46.62-mRNA	1.41075269
Insect	epiGBS	GO:0000027	ribosomal large subunit assembly	0.04599149	0.04599149	0.70563314	AT3G55280.	BP	maker-scaffold_7-snap-gene-15.119-mRN	2.91879867
Insect	epiGBS	GO:0055086	nucleobase-containing small molecule m	0.04703866	0.04703866	0.70563314	AT3G21070.	BP	maker-scaffold_9-augustus-gene-14.82-m	1.38841045
Insect	epiGBS	GO:0010629	negative regulation of gene expression	0.04934862	0.04934862	0.70563314	AT1G08620.	BP	maker-scaffold_24-augustus-gene-3.78-m	1.41075269
Insect	epiGBS	GO:0007018	microtubule-based movement	0.04993832	0.04993832	0.70563314	AT2G36200.	BP	maker-scaffold_8-snap-gene-26.106-mRN	2.0863244
Insect	epiGBS	GO:0016773	phosphotransferase activity, alcohol gro	0.00025293	0.00025293	0.15708214	AT4G27290.	MF	maker-scaffold_10-augustus-gene-42.45-	1.45078911
Insect	epiGBS	GO:0004674	protein serine/threonine kinase activity	0.00057121	0.00057121	0.17554945	AT4G27290.	MF	maker-scaffold_10-augustus-gene-42.45-	1.51210291
Insect	epiGBS	GO:0140098	catalytic activity, acting on RNA	0.00113669	0.00113669	0.17554945	AT1G55870.	MF	maker-scaffold_10-augustus-gene-17.55-	1.71970943
Insect	epiGBS	GO:0016301	kinase activity	0.00159397	0.00159397	0.17554945	AT4G27290.	MF	maker-scaffold_10-augustus-gene-42.45-	1.35920782
Insect	epiGBS	GO:0016713	oxidoreductase activity, acting on paired	0.00171942	0.00171942	0.17554945	AT2G45970.	MF	augustus_masked-scaffold_3-processed-g	7.14953271

stress	technique	ID	Description	pvalue	p.adjust	qvalue	geneID	bgsource	PN_annoated_genes	foldEnrichme
Insect	epiGBS	GO:0034033	purine nucleoside bisphosphate biosynth	0.01341449	0.01341449	0.53183513	AT1G09430.	BP	maker-scaffold_44-snap-gene-9.114-mRN	3.02304148
Insect	epiGBS	GO:0018193	peptidyl-amino acid modification	0.01354679	0.01354679	0.53183513	AT3G53570.	BP	maker-scaffold_17-augustus-gene-25.64-	1.45412122
Insect	epiGBS	GO:0050829	defense response to Gram-negative bac	0.01423688	0.01423688	0.53183513	AT3G14310.	BP	maker-scaffold_23-snap-gene-6.77-mRNA	3.41311134
Insect	epiGBS	GO:0009910	negative regulation of flower developme	0.01498425	0.01498425	0.53183513	AT1G30970.	BP	snap_masked-scaffold_6-processed-gene	2.95273819
Insect	epiGBS	GO:0002239	response to oomycetes	0.01518693	0.01518693	0.53183513	AT1G19100.	BP	maker-scaffold_21-snap-gene-12.84-mRN	2.20430108
Insect	epiGBS	GO:0019439	aromatic compound catabolic process	0.01534611	0.01534611	0.53183513	AT5G54080.	BP	maker-scaffold_10-augustus-gene-20.65-	1.60718661
Insect	epiGBS	GO:0006801	superoxide metabolic process	0.01557889	0.01557889	0.53183513	AT2G28190.	BP	maker-scaffold_7-augustus-gene-2.65-mR	4.03072197
Insect	epiGBS	GO:0044273	sulfur compound catabolic process	0.01557889	0.01557889	0.53183513	AT1G64660.	BP	augustus_masked-scaffold_6-processed-g	4.03072197
Insect	epiGBS	GO:0000070	mitotic sister chromatid segregation	0.01576582	0.01576582	0.53183513	AT5G47690.	BP	maker-scaffold_4-snap-gene-69.82-mRNA	2.45348294
Insect	epiGBS	GO:0006898	receptor-mediated endocytosis	0.0158657	0.0158657	0.53183513	AT2G40060.	BP	maker-scaffold_11-augustus-gene-20.76-	2.64516129
Insect	epiGBS	GO:2001020	regulation of response to DNA damage s	0.01623401	0.01623401	0.53183513	AT1G79950.	BP	maker-scaffold_6-snap-gene-59.48-mRNA	3.30645161
Insect	epiGBS	GO:0016197	endosomal transport	0.01627125	0.01627125	0.53183513	AT2G06530.	BP	maker-scaffold_6-augustus-gene-54.60-m	2.29459775
Insect	epiGBS	GO:1902275	regulation of chromatin organization	0.01627125	0.01627125	0.53183513	AT1G19100.	BP	maker-scaffold_21-snap-gene-12.84-mRN	2.29459775
Insect	epiGBS	GO:0006397	mRNA processing	0.0164466	0.0164466	0.53183513	AT1G55870.	BP	maker-scaffold_10-augustus-gene-17.55-	1.52277275
Insect	epiGBS	GO:0015937	coenzyme A biosynthetic process	0.01679404	0.01679404	0.53183513	AT4G35360.	BP	maker-scaffold_16-augustus-gene-9.109-	5.29032258
Insect	epiGBS	GO:0045144	meiotic sister chromatid segregation	0.01679404	0.01679404	0.53183513	AT5G05510.	BP	maker-scaffold_5-augustus-gene-45.107-	5.29032258
Insect	epiGBS	GO:0051177	meiotic sister chromatid cohesion	0.01679404	0.01679404	0.53183513	AT5G05510.	BP	maker-scaffold_5-augustus-gene-45.107-	5.29032258
Insect	epiGBS	GO:0051128	regulation of cellular component organiz	0.01682025	0.01682025	0.53183513	AT1G79950.	BP	maker-scaffold_6-snap-gene-59.48-mRNA	1.50793043
Insect	epiGBS	GO:0000165	MAPK cascade	0.01686554	0.01686554	0.53183513	AT3G58640.	BP	maker-scaffold_38-snap-gene-3.55-mRNA	2.07834101
Insect	epiGBS	GO:0006897	endocytosis	0.01686554	0.01686554	0.53183513	AT2G40060.	BP	maker-scaffold_11-augustus-gene-20.76-	2.07834101
Insect	epiGBS	GO:0000819	sister chromatid segregation	0.01748646	0.01748646	0.53804288	AT5G47690.	BP	maker-scaffold_4-snap-gene-69.82-mRNA	2.26728111
Insect	epiGBS	GO:0009648	photoperiodism	0.0175499	0.0175499	0.53804288	AT1G72390.	BP	maker-scaffold_6-snap-gene-51.77-mRNA	1.79143199
Insect	epiGBS	GO:2000026	regulation of multicellular organismal d	0.01783658	0.01783658	0.53934125	AT5G60910.	BP	maker-scaffold_9-snap-gene-46.62-mRNA	1.50080073
Insect	epiGBS	GO:0048511	rhythmic process	0.01814044	0.01814044	0.5411167	AT1G72390.	BP	maker-scaffold_6-snap-gene-51.77-mRNA	1.82424917
Insect	epiGBS	GO:0043484	regulation of RNA splicing	0.01840903	0.01840903	0.54180695	AT5G62600.	BP	maker-scaffold_21-augustus-gene-4.64-m	3.20625611
Insect	epiGBS	GO:0002229	defense response to oomycetes	0.0187665	0.0187665	0.54506022	AT1G19100.	BP	maker-scaffold_21-snap-gene-12.84-mRN	2.24060721
Insect	epiGBS	GO:0006401	RNA catabolic process	0.01956694	0.01956694	0.55389958	AT3G60500.	BP	maker-scaffold_7-snap-gene-23.104-mRN	1.8516129
Insect	epiGBS	GO:0018022	peptidyl-lysine methylation	0.0201618	0.0201618	0.55389958	AT1G66680.	BP	maker-scaffold_19-augustus-gene-22.40-	2.02412342
Insect	epiGBS	GO:0140013	meiotic nuclear division	0.0201618	0.0201618	0.55389958	AT3G18524.	BP	maker-scaffold_31-augustus-gene-3.35-m	2.02412342
Insect	epiGBS	GO:0034968	histone lysine methylation	0.02100333	0.02100333	0.55389958	AT5G06620.	BP	maker-scaffold_8-augustus-gene-32.87-m	2.09517726
Insect	epiGBS	GO:0006388	tRNA splicing, via endonucleolytic cleava	0.0210783	0.0210783	0.55389958	AT1G07910.	BP	augustus_masked-scaffold_17-processed-	4.88337469
Insect	epiGBS	GO:0006493	protein O-linked glycosylation	0.0210783	0.0210783	0.55389958	AT3G04240.	BP	maker-scaffold_65-snap-gene-0.56-mRNA	4.88337469
Insect	epiGBS	GO:0031126	snoRNA 3'-end processing	0.0210783	0.0210783	0.55389958	AT5G52470.	BP	maker-scaffold_25-snap-gene-18.70-mRN	4.88337469
Insect	epiGBS	GO:0032506	cytokinetic process	0.0210783	0.0210783	0.55389958	AT5G18700.	BP	maker-scaffold_25-augustus-gene-20.67-	4.88337469
Insect	epiGBS	GO:1903046	meiotic cell cycle process	0.0215394	0.0215394	0.55539676	AT3G18524.	BP	maker-scaffold_31-augustus-gene-3.35-m	1.82875349
Insect	epiGBS	GO:0072521	purine-containing compound metabolic p	0.02163849	0.02163849	0.55539676	AT1G09430.	BP	maker-scaffold_44-snap-gene-9.114-mRN	1.63407647
Insect	epiGBS	GO:0034660	ncRNA metabolic process	0.02325513	0.02325513	0.59003036	AT5G16750.	BP	augustus_masked-scaffold_24-processed-	1.43038543
Insect	epiGBS	GO:0016071	mRNA metabolic process	0.02462009	0.02462009	0.60242851	AT1G55870.	BP	maker-scaffold_10-augustus-gene-17.55-	1.40814501
Insect	epiGBS	GO:0001510	RNA methylation	0.02490876	0.02490876	0.60242851	AT3G27180.	BP	maker-scaffold_9-snap-gene-48.74-mRNA	2.2572043
Insect	epiGBS	GO:0032787	monocarboxylic acid metabolic process	0.02537395	0.02537395	0.60242851	AT5G54080.	BP	maker-scaffold_10-augustus-gene-20.65-	1.36014273
Insect	epiGBS	GO:0006271	DNA strand elongation involved in DNA r	0.02590293	0.02590293	0.60242851	AT1G08130.	BP	maker-scaffold_7-snap-gene-2.78-mRNA-	4.53456221
Insect	epiGBS	GO:0015918	sterol transport	0.02590293	0.02590293	0.60242851	AT1G13170.	BP	maker-scaffold_25-augustus-gene-14.54-	4.53456221
Insect	epiGBS	GO:0022616	DNA strand elongation	0.02590293	0.02590293	0.60242851	AT1G08130.	BP	maker-scaffold_7-snap-gene-2.78-mRNA-	4.53456221
Insect	epiGBS	GO:1902288	regulation of defense response to oomyc	0.02590293	0.02590293	0.60242851	AT1G19100.	BP	maker-scaffold_21-snap-gene-12.84-mRN	4.53456221
Insect	epiGBS	GO:0007623	circadian rhythm	0.02592712	0.02592712	0.60242851	AT1G72390.	BP	maker-scaffold_6-snap-gene-51.77-mRNA	1.78468714
Insect	epiGBS	GO:0098813	nuclear chromosome segregation	0.02667664	0.02667664	0.61338726	AT5G47690.	BP	maker-scaffold_4-snap-gene-69.82-mRNA	1.93978495
Insect	epiGBS	GO:0033043	regulation of organelle organization	0.02818286	0.02818286	0.63286377	AT1G79950.	BP	maker-scaffold_6-snap-gene-59.48-mRNA	1.63477779
Insect	epiGBS	GO:0048573	photoperiodism, flowering	0.02835405	0.02835405	0.63286377	AT1G72390.	BP	maker-scaffold_6-snap-gene-51.77-mRNA	1.76344086
Insect	epiGBS	GO:0042147	retrograde transport, endosome to Golg	0.0283838	0.0283838	0.63286377	AT2G44610.	BP	augustus_masked-scaffold_6-processed-g	3.38580645
Insect	epiGBS	GO:0034655	nucleobase-containing compound catabo	0.02920564	0.02920564	0.63355117	AT3G60500.	BP	maker-scaffold_7-snap-gene-23.104-mRN	1.68448082

stress	technique	ID	Description	pvalue	p.adjust	qvalue	geneID	bgsource	PN_annoated_genes	foldEnrichme
Insect	epiGBS	GO:0046912	transferase activity, transferring acyl grc	0.00171942	0.00171942	0.17554945	AT2G44350.	MF	augustus_masked-scaffold_9-processed-g	7.14953271
Insect	epiGBS	GO:0016627	oxidoreductase activity, acting on the CH	0.00197865	0.00197865	0.17554945	AT1G75280.	MF	maker-scaffold_14-snap-gene-31.111-mR	2.51199798
Insect	epiGBS	GO:0004672	protein kinase activity	0.00230975	0.00230975	0.17930955	AT4G27290.	MF	maker-scaffold_10-augustus-gene-42.45-	1.39349979
Insect	epiGBS	GO:0016407	acetyltransferase activity	0.00278439	0.00278439	0.19213895	AT5G58600.	MF	maker-scaffold_9-augustus-gene-23.108-	2.32775484
Insect	epiGBS	GO:0003916	DNA topoisomerase activity	0.00322703	0.00322703	0.19961178	AT5G04130.	MF	maker-scaffold_4-snap-gene-59.57-mRNA	6.1281709
Insect	epiGBS	GO:0016772	transferase activity, transferring phosph	0.00432207	0.00432207	0.19961178	AT4G27290.	MF	maker-scaffold_10-augustus-gene-42.45-	1.29555291
Insect	epiGBS	GO:0019829	ATPase-coupled cation transmembrane	0.00506604	0.00506604	0.19961178	AT4G11150.	MF	maker-scaffold_24-augustus-gene-5.51-m	2.7576769
Insect	epiGBS	GO:0140097	catalytic activity, acting on DNA	0.00510494	0.00510494	0.19961178	AT1G79950.	MF	maker-scaffold_6-snap-gene-59.48-mRNA	1.88145598
Insect	epiGBS	GO:0042054	histone methyltransferase activity	0.00510579	0.00510579	0.19961178	AT5G06620.	MF	maker-scaffold_8-augustus-gene-32.87-m	3.26391711
Insect	epiGBS	GO:0003951	NAD+ kinase activity	0.00544685	0.00544685	0.19961178	AT3G21070.	MF	maker-scaffold_9-augustus-gene-14.82-m	5.36214953
Insect	epiGBS	GO:0008094	DNA-dependent ATPase activity	0.00557246	0.00557246	0.19961178	AT1G79950.	MF	maker-scaffold_6-snap-gene-59.48-mRNA	2.23065421
Insect	epiGBS	GO:0015662	ion transmembrane transporter activity,	0.00610677	0.00610677	0.19961178	AT4G11150.	MF	maker-scaffold_24-augustus-gene-5.51-m	2.68107477
Insect	epiGBS	GO:0032182	ubiquitin-like protein binding	0.00610677	0.00610677	0.19961178	AT3G18860.	MF	maker-scaffold_7-snap-gene-43.123-mRN	2.68107477
Insect	epiGBS	GO:0140358	P-type transmembrane transporter activ	0.00610677	0.00610677	0.19961178	AT4G11150.	MF	maker-scaffold_24-augustus-gene-5.51-m	2.68107477
Insect	epiGBS	GO:0004386	helicase activity	0.00699425	0.00699425	0.21718999	AT1G79950.	MF	maker-scaffold_6-snap-gene-59.48-mRNA	1.9395009
Insect	epiGBS	GO:0015248	sterol transporter activity	0.00947291	0.00947291	0.2711382	AT1G13170.	MF	maker-scaffold_25-augustus-gene-14.54-	6.43457944
Insect	epiGBS	GO:0004712	protein serine/threonine/tyrosine kinase	0.01039376	0.01039376	0.2711382	AT3G53570.	MF	maker-scaffold_17-augustus-gene-25.64-	2.63982746
Insect	epiGBS	GO:0003724	RNA helicase activity	0.01047788	0.01047788	0.2711382	AT1G79950.	MF	maker-scaffold_6-snap-gene-59.48-mRNA	2.22579792
Insect	epiGBS	GO:0008186	RNA-dependent ATPase activity	0.01047788	0.01047788	0.2711382	AT1G79950.	MF	maker-scaffold_6-snap-gene-59.48-mRNA	2.22579792
Insect	epiGBS	GO:0043130	ubiquitin binding	0.01135843	0.01135843	0.28216742	AT3G18860.	MF	maker-scaffold_7-snap-gene-43.123-mRN	2.59983008
Insect	epiGBS	GO:0008173	RNA methyltransferase activity	0.01483496	0.01483496	0.33027624	AT3G27180.	MF	maker-scaffold_9-snap-gene-48.74-mRNA	2.68107477
Insect	epiGBS	GO:0009881	photoreceptor activity	0.01489042	0.01489042	0.33027624	AT5G57360.	MF	maker-scaffold_8-augustus-gene-50.34-m	4.08544726
Insect	epiGBS	GO:0032050	clathrin heavy chain binding	0.01489042	0.01489042	0.33027624	AT2G40060.	MF	maker-scaffold_11-augustus-gene-20.76-	4.08544726
Insect	epiGBS	GO:0016730	oxidoreductase activity, acting on iron-su	0.01619887	0.01619887	0.33645876	AT5G59400.	MF	maker-scaffold_7-snap-gene-18.95-mRNA	5.36214953
Insect	epiGBS	GO:0008080	N-acetyltransferase activity	0.01625267	0.01625267	0.33645876	AT2G39020.	MF	augustus_masked-scaffold_11-processed-	2.63403837
Insect	epiGBS	GO:0016410	N-acyltransferase activity	0.01858104	0.01858104	0.36093385	AT2G39020.	MF	augustus_masked-scaffold_11-processed-	2.38317757
Insect	epiGBS	GO:0008170	N-methyltransferase activity	0.01993053	0.01993053	0.36093385	AT1G66680.	MF	maker-scaffold_19-augustus-gene-22.40-	2.2188205
Insect	epiGBS	GO:0000339	RNA cap binding	0.02034076	0.02034076	0.36093385	AT5G18410.	MF	maker-scaffold_65-snap-gene-0.58-mRNA	4.94967649
Insect	epiGBS	GO:0032934	sterol binding	0.02034076	0.02034076	0.36093385	AT1G13170.	MF	maker-scaffold_25-augustus-gene-14.54-	4.94967649
Insect	epiGBS	GO:0070402	NADPH binding	0.02034076	0.02034076	0.36093385	AT1G75280.	MF	maker-scaffold_14-snap-gene-31.111-mR	4.94967649
Insect	epiGBS	GO:0008276	protein methyltransferase activity	0.02492676	0.02492676	0.42747949	AT1G66680.	MF	maker-scaffold_19-augustus-gene-22.40-	2.25774717
Insect	epiGBS	GO:0045330	aspartyl esterase activity	0.0256058	0.0256058	0.42747949	AT3G14310.	MF	maker-scaffold_23-snap-gene-6.77-mRNA	2.62635896
Insect	epiGBS	GO:0022853	active ion transmembrane transporter a	0.02633576	0.02633576	0.42747949	AT2G38290.	MF	augustus_masked-scaffold_9-processed-g	1.60064165
Insect	epiGBS	GO:0018024	histone-lysine N-methyltransferase activ	0.02756383	0.02756383	0.42747949	AT5G06620.	MF	maker-scaffold_8-augustus-gene-32.87-m	2.89845921
Insect	epiGBS	GO:0030599	pectinesterase activity	0.02799064	0.02799064	0.42747949	AT3G14310.	MF	maker-scaffold_23-snap-gene-6.77-mRNA	2.57383178
Insect	epiGBS	GO:0043138	3'-5' DNA helicase activity	0.03020025	0.03020025	0.42747949	AT5G61140.	MF	maker-scaffold_19-snap-gene-25.62-mRN	4.28971963
Insect	epiGBS	GO:0003678	DNA helicase activity	0.03051745	0.03051745	0.42747949	AT1G79950.	MF	maker-scaffold_6-snap-gene-59.48-mRNA	2.52336449
Insect	epiGBS	GO:0016278	lysine N-methyltransferase activity	0.03051745	0.03051745	0.42747949	AT1G66680.	MF	maker-scaffold_19-augustus-gene-22.40-	2.52336449
Insect	epiGBS	GO:0016279	protein-lysine N-methyltransferase activ	0.03051745	0.03051745	0.42747949	AT1G66680.	MF	maker-scaffold_19-augustus-gene-22.40-	2.52336449
Insect	epiGBS	GO:0051117	ATPase binding	0.03097415	0.03097415	0.42747949	AT4G39080.	MF	maker-scaffold_12-snap-gene-8.134-mRN	3.29978433
Insect	epiGBS	GO:0016746	transferase activity, transferring acyl grc	0.03170612	0.03170612	0.42806894	AT5G58600.	MF	maker-scaffold_9-augustus-gene-23.108-	1.45062983
Insect	epiGBS	GO:0003684	damaged DNA binding	0.03375971	0.03375971	0.4460969	AT3G18524.	MF	maker-scaffold_31-augustus-gene-3.35-m	2.74982027
Insect	epiGBS	GO:0008641	ubiquitin-like modifier activating enzym	0.03591257	0.03591257	0.46151781	AT5G06460.	MF	maker-scaffold_9-snap-gene-24.107-mRN	4.02161215
Insect	epiGBS	GO:0031267	small GTPase binding	0.0371561	0.0371561	0.46151781	AT5G62600.	MF	maker-scaffold_21-augustus-gene-4.64-m	2.68107477
Insect	epiGBS	GO:1901682	sulfur compound transmembrane transp	0.0371561	0.0371561	0.46151781	AT4G39850.	MF	maker-scaffold_14-augustus-gene-19.66-	2.68107477
Insect	epiGBS	GO:0034212	peptide N-acetyltransferase activity	0.03944609	0.03944609	0.46947029	AT3G01460.	MF	maker-scaffold_27-augustus-gene-3.53-m	3.06408545
Insect	epiGBS	GO:0000175	3'-5'-exoribonuclease activity	0.04075456	0.04075456	0.46947029	AT1G55870.	MF	maker-scaffold_10-augustus-gene-17.55-	2.6156827
Insect	epiGBS	GO:0042626	ATPase-coupled transmembrane transpo	0.0411678	0.0411678	0.46947029	AT3G04340.	MF	maker-scaffold_24-snap-gene-4.88-mRNA	1.56305573
Insect	epiGBS	GO:0071949	FAD binding	0.04186015	0.04186015	0.46947029	AT5G44440.	MF	snap_masked-scaffold_22-processed-gen	2.04272363
Insect	epiGBS	GO:0017056	structural constituent of nuclear pore	0.04213737	0.04213737	0.46947029	AT2G41620.	MF	maker-scaffold_31-augustus-gene-16.62-	3.78504673

stress	technique	ID	Description	pvalue	p.adjust	qvalue	geneID	bgsource	PN_annoated_genes	foldEnrichme
Artificial	epiGBS	GO:0008574	ATP-dependent microtubule motor activ	0.02243042	0.02243042	0.36049916	AT2G36200.	MF	maker-scaffold_8-snap-gene-26.106-mRN	3.59225201
Artificial	epiGBS	GO:0008649	rRNA methyltransferase activity	0.02243042	0.02243042	0.36049916	AT5G57280.	MF	augustus_masked-scaffold_9-processed-g	3.59225201
Artificial	epiGBS	GO:0140296	general transcription initiation factor bir	0.02243042	0.02243042	0.36049916	AT1G55520.	MF	maker-scaffold_24-augustus-gene-8.91-m	3.59225201
Artificial	epiGBS	GO:0015662	ion transmembrane transporter activity,	0.02432766	0.02432766	0.37157232	AT4G11150.	MF	maker-scaffold_24-augustus-gene-5.51-m	2.13289963
Artificial	epiGBS	GO:0140358	P-type transmembrane transporter activ	0.02432766	0.02432766	0.37157232	AT4G11150.	MF	maker-scaffold_24-augustus-gene-5.51-m	2.13289963
Artificial	epiGBS	GO:0016796	exonuclease activity, active with either r	0.02765228	0.02765228	0.37157232	AT1G55870.	MF	maker-scaffold_10-augustus-gene-17.55-	2.20170284
Artificial	epiGBS	GO:0140104	molecular carrier activity	0.02765228	0.02765228	0.37157232	AT2G28800.	MF	maker-scaffold_9-snap-gene-6.79-mRNA-	2.20170284
Artificial	epiGBS	GO:0004386	helicase activity	0.02773701	0.02773701	0.37157232	AT3G18600.	MF	maker-scaffold_9-augustus-gene-11.70-m	1.63371035
Artificial	epiGBS	GO:0016810	hydrolase activity, acting on carbon-nitrc	0.02817937	0.02817937	0.37157232	AT1G08980.	MF	maker-scaffold_44-augustus-gene-4.85-m	1.80342733
Artificial	epiGBS	GO:0015079	potassium ion transmembrane transport	0.02850794	0.02850794	0.37157232	AT2G26650.	MF	maker-scaffold_4-augustus-gene-4.36-mR	2.07525369
Artificial	epiGBS	GO:0030515	snoRNA binding	0.02875676	0.02875676	0.37157232	AT5G16750.	MF	augustus_masked-scaffold_24-processed-	2.84386617
Artificial	epiGBS	GO:0047262	polygalacturonate 4-alpha-galacturonos	0.02875676	0.02875676	0.37157232	AT1G18580.	MF	augustus_masked-scaffold_19-processed-	2.84386617
Artificial	epiGBS	GO:0016713	oxidoreductase activity, acting on paired	0.02965457	0.02965457	0.37157232	AT2G27690.	MF	augustus_masked-scaffold_24-processed-	4.26579926
Artificial	epiGBS	GO:0051539	4 iron, 4 sulfur cluster binding	0.03014035	0.03014035	0.37157232	AT2G43360.	MF	maker-scaffold_14-augustus-gene-31.94-	1.89591078
Artificial	epiGBS	GO:0008092	cytoskeletal protein binding	0.03097397	0.03097397	0.37157232	AT2G41740.	MF	maker-scaffold_17-snap-gene-14.101-mR	1.46255975
Artificial	epiGBS	GO:0000175	3'-5'-exoribonuclease activity	0.03101585	0.03101585	0.37157232	AT1G55870.	MF	maker-scaffold_10-augustus-gene-17.55-	2.49705322
Artificial	epiGBS	GO:0004842	ubiquitin-protein transferase activity	0.03109181	0.03109181	0.37157232	AT5G06460.	MF	maker-scaffold_9-snap-gene-24.107-mRN	1.40420325
Artificial	epiGBS	GO:0140102	catalytic activity, acting on a rRNA	0.03157771	0.03157771	0.37157232	AT5G57280.	MF	augustus_masked-scaffold_9-processed-g	3.25013277
Artificial	epiGBS	GO:0003729	mRNA binding	0.03283377	0.03283377	0.37957415	AT1G53210.	MF	maker-scaffold_10-augustus-gene-21.41-	1.23387533
Artificial	epiGBS	GO:0042626	ATPase-coupled transmembrane transpo	0.0334684	0.0334684	0.38023987	AT2G28800.	MF	maker-scaffold_9-snap-gene-6.79-mRNA-	1.51979893
Artificial	epiGBS	GO:0005516	calmodulin binding	0.03453765	0.03453765	0.383247	AT4G27290.	MF	maker-scaffold_10-augustus-gene-42.45-	1.45935238
Artificial	epiGBS	GO:0004712	protein serine/threonine/tyrosine kinase	0.03546214	0.03546214	0.383247	AT1G08720.	MF	snap_masked-scaffold_28-processed-gen	2.10008579
Artificial	epiGBS	GO:0022821	potassium ion antiporter activity	0.03685845	0.03685845	0.383247	AT3G05030.	MF	augustus_masked-scaffold_28-processed-	3.10239946
Artificial	epiGBS	GO:0004659	prenyltransferase activity	0.03693513	0.03693513	0.383247	AT5G47770.	MF	maker-scaffold_4-augustus-gene-70.122-	2.66612454
Artificial	epiGBS	GO:0016772	transferase activity, transferring phosph	0.03718823	0.03718823	0.383247	AT4G27290.	MF	maker-scaffold_10-augustus-gene-42.45-	1.17641322
Artificial	epiGBS	GO:0015399	primary active transmembrane transpor	0.03722271	0.03722271	0.383247	AT2G28800.	MF	maker-scaffold_9-snap-gene-6.79-mRNA-	1.46089016
Artificial	epiGBS	GO:0003774	motor activity	0.03832843	0.03832843	0.38856028	AT2G36200.	MF	maker-scaffold_8-snap-gene-26.106-mRN	1.96883043
Artificial	epiGBS	GO:0016773	phosphotransferase activity, alcohol gro	0.04301828	0.04301828	0.42342294	AT4G27290.	MF	maker-scaffold_10-augustus-gene-42.45-	1.19350571
Artificial	epiGBS	GO:0015298	solute:cation antiporter activity	0.04421794	0.04421794	0.42342294	AT1G53210.	MF	maker-scaffold_10-augustus-gene-21.41-	2.13289963
Artificial	epiGBS	GO:0000146	microfilament motor activity	0.04498023	0.04498023	0.42342294	AT4G33200.	MF	maker-scaffold_8-snap-gene-22.92-mRNA	3.65639936
Artificial	epiGBS	GO:0003916	DNA topoisomerase activity	0.04498023	0.04498023	0.42342294	AT5G55310.	MF	maker-scaffold_10-augustus-gene-13.32-	3.65639936
Artificial	epiGBS	GO:0019239	deaminase activity	0.04498023	0.04498023	0.42342294	AT2G38280.	MF	maker-scaffold_4-augustus-gene-33.62-m	3.65639936
Artificial	epiGBS	GO:0008757	S-adenosylmethionine-dependent methy	0.04619176	0.04619176	0.42470083	AT5G57280.	MF	augustus_masked-scaffold_9-processed-g	1.55953951
Artificial	epiGBS	GO:0015081	sodium ion transmembrane transporter	0.04640501	0.04640501	0.42470083	AT1G53210.	MF	maker-scaffold_10-augustus-gene-21.41-	2.50929368
Artificial	epiGBS	GO:0031981	nuclear lumen	4.11E-05	4.11E-05	0.01498392	AT3G13224.	CC	maker-scaffold_10-augustus-gene-18.65-	1.5264291
Artificial	epiGBS	GO:0032040	small-subunit processome	0.00019751	0.00019751	0.02225493	AT5G16750.	CC	augustus_masked-scaffold_24-processed-	3.55913924
Artificial	epiGBS	GO:0031974	membrane-enclosed lumen	0.00030552	0.00030552	0.02225493	AT3G13224.	CC	maker-scaffold_10-augustus-gene-18.65-	1.40440265
Artificial	epiGBS	GO:0043233	organelle lumen	0.00030552	0.00030552	0.02225493	AT3G13224.	CC	maker-scaffold_10-augustus-gene-18.65-	1.40440265
Artificial	epiGBS	GO:0070013	intracellular organelle lumen	0.00030552	0.00030552	0.02225493	AT3G13224.	CC	maker-scaffold_10-augustus-gene-18.65-	1.40440265
Artificial	epiGBS	GO:1990904	ribonucleoprotein complex	0.00112526	0.00112526	0.06830504	AT1G44910.	CC	maker-scaffold_9-snap-gene-28.97-mRNA	1.48809038
Artificial	epiGBS	GO:0005681	spliceosomal complex	0.0015322	0.0015322	0.07972045	AT1G44910.	CC	maker-scaffold_9-snap-gene-28.97-mRNA	2.07716319
Artificial	epiGBS	GO:0034518	RNA cap binding complex	0.00195166	0.00195166	0.0844513	AT5G44200.	CC	maker-scaffold_53-augustus-gene-3.69-m	6.73000875
Artificial	epiGBS	GO:0071004	U2-type prespliceosome	0.00231875	0.00231875	0.0844513	AT1G44910.	CC	maker-scaffold_9-snap-gene-28.97-mRNA	4.20625547
Artificial	epiGBS	GO:0071010	prespliceosome	0.00231875	0.00231875	0.0844513	AT1G44910.	CC	maker-scaffold_9-snap-gene-28.97-mRNA	4.20625547
Artificial	epiGBS	GO:0016607	nuclear speck	0.00440035	0.00440035	0.13610173	AT1G24706.	CC	maker-scaffold_24-snap-gene-23.45-mRN	2.27838838
Artificial	epiGBS	GO:0005667	transcription regulator complex	0.00475612	0.00475612	0.13610173	AT1G54140.	CC	maker-scaffold_6-augustus-gene-52.52-m	2.18102135
Artificial	epiGBS	GO:0043228	non-membrane-bounded organelle	0.00595447	0.00595447	0.13610173	AT5G55310.	CC	maker-scaffold_10-augustus-gene-13.32-	1.22618587
Artificial	epiGBS	GO:0043232	intracellular non-membrane-bounded or	0.00595447	0.00595447	0.13610173	AT5G55310.	CC	maker-scaffold_10-augustus-gene-13.32-	1.22618587
Artificial	epiGBS	GO:0030686	90S preribosome	0.00634895	0.00634895	0.13610173	AT5G16750.	CC	augustus_masked-scaffold_24-processed-	3.48103901
Artificial	epiGBS	GO:0005654	nucleoplasm	0.00663717	0.00663717	0.13610173	AT3G13224.	CC	maker-scaffold_10-augustus-gene-18.65-	1.52226388

stress	technique	ID	Description	pvalue	p.adjust	qvalue	geneID	bgsource	PN_annoated_genes	foldEnrichme
Insect	epiGBS	GO:0051276	chromosome organization	1.25E-06	1.25E-06	0.00275274	AT1G79950.:	BP	maker-scaffold_6-snap-gene-59.48-mRNA	1.94234066
Insect	epiGBS	GO:0016570	histone modification	0.00014953	0.00014953	0.1650337	AT5G46910.:	BP	maker-scaffold_6-augustus-gene-37.101-	2.27744829
Insect	epiGBS	GO:0006325	chromatin organization	0.00100231	0.00100231	0.44294461	AT5G46910.:	BP	maker-scaffold_6-augustus-gene-37.101-	1.74680463
Insect	epiGBS	GO:0044270	cellular nitrogen compound catabolic pr	0.00106128	0.00106128	0.44294461	AT3G60500.:	BP	maker-scaffold_7-snap-gene-23.104-mRN	1.99165085
Insect	epiGBS	GO:0016571	histone methylation	0.00135146	0.00135146	0.44294461	AT2G19390.:	BP	maker-scaffold_23-snap-gene-4.66-mRNA	2.51066156
Insect	epiGBS	GO:0006996	organelle organization	0.00137396	0.00137396	0.44294461	AT1G79950.:	BP	maker-scaffold_6-snap-gene-59.48-mRNA	1.3040878
Insect	epiGBS	GO:0016310	phosphorylation	0.00160693	0.00160693	0.44294461	AT4G27290.:	BP	maker-scaffold_10-augustus-gene-42.45-	1.33594005
Insect	epiGBS	GO:0006265	DNA topological change	0.001807	0.001807	0.44294461	AT5G04130.:	BP	maker-scaffold_4-snap-gene-59.57-mRNA	7.05376344
Insect	epiGBS	GO:0046700	heterocycle catabolic process	0.00199675	0.00199675	0.44294461	AT3G60500.:	BP	maker-scaffold_7-snap-gene-23.104-mRN	1.93138761
Insect	epiGBS	GO:0009056	catabolic process	0.00200666	0.00200666	0.44294461	AT5G54080.:	BP	maker-scaffold_10-augustus-gene-20.65-	1.29005817
Insect	epiGBS	GO:0044272	sulfur compound biosynthetic process	0.00240144	0.00240144	0.46229123	AT1G64660.:	BP	augustus_masked-scaffold_6-processed-g	2.15414333
Insect	epiGBS	GO:0046113	nucleobase catabolic process	0.00251317	0.00251317	0.46229123	AT5G12200.:	BP	maker-scaffold_9-augustus-gene-21.101-	6.51116625
Insect	epiGBS	GO:0031123	RNA 3'-end processing	0.00300446	0.00300446	0.46249534	AT3G60500.:	BP	maker-scaffold_7-snap-gene-23.104-mRN	2.97580645
Insect	epiGBS	GO:1901575	organic substance catabolic process	0.00309198	0.00309198	0.46249534	AT5G54080.:	BP	maker-scaffold_10-augustus-gene-20.65-	1.29785951
Insect	epiGBS	GO:0008380	RNA splicing	0.00314285	0.00314285	0.46249534	AT3G16010.:	BP	maker-scaffold_6-snap-gene-59.54-mRNA	1.78634269
Insect	epiGBS	GO:0033044	regulation of chromosome organization	0.003605	0.003605	0.4795323	AT1G79950.:	BP	maker-scaffold_6-snap-gene-59.48-mRNA	2.71298594
Insect	epiGBS	GO:2000242	negative regulation of reproductive proc	0.00369311	0.00369311	0.4795323	AT1G30970.:	BP	snap_masked-scaffold_6-processed-gene-	2.55795817
Insect	epiGBS	GO:0044248	cellular catabolic process	0.00399625	0.00399625	0.49006691	AT5G54080.:	BP	maker-scaffold_10-augustus-gene-20.65-	1.30474237
Insect	epiGBS	GO:0034614	cellular response to reactive oxygen spe	0.00484729	0.00484729	0.51683496	AT2G28190.:	BP	maker-scaffold_7-augustus-gene-2.65-mR	3.29175627
Insect	epiGBS	GO:0006396	RNA processing	0.00512402	0.00512402	0.51683496	AT1G55870.:	BP	maker-scaffold_10-augustus-gene-17.55-	1.40140996
Insect	epiGBS	GO:0018205	peptidyl-lysine modification	0.00520201	0.00520201	0.51683496	AT3G01460.:	BP	maker-scaffold_27-augustus-gene-3.53-m	1.95334988
Insect	epiGBS	GO:0043414	macromolecule methylation	0.00558161	0.00558161	0.51683496	AT3G27180.:	BP	maker-scaffold_9-snap-gene-48.74-mRNA	1.80445111
Insect	epiGBS	GO:0006282	regulation of DNA repair	0.00561938	0.00561938	0.51683496	AT1G79950.:	BP	maker-scaffold_6-snap-gene-59.48-mRNA	4.23225807
Insect	epiGBS	GO:0034661	ncRNA catabolic process	0.00561938	0.00561938	0.51683496	AT3G60500.:	BP	maker-scaffold_7-snap-gene-23.104-mRN	4.23225807
Insect	epiGBS	GO:0072583	clathrin-dependent endocytosis	0.00619298	0.00619298	0.51696471	AT2G40060.:	BP	maker-scaffold_11-augustus-gene-20.76-	3.15168154
Insect	epiGBS	GO:0006479	protein methylation	0.00632339	0.00632339	0.51696471	AT1G66680.:	BP	maker-scaffold_19-augustus-gene-22.40-	2.06116464
Insect	epiGBS	GO:0008213	protein alkylation	0.00632339	0.00632339	0.51696471	AT1G66680.:	BP	maker-scaffold_19-augustus-gene-22.40-	2.06116464
Insect	epiGBS	GO:0006468	protein phosphorylation	0.00690496	0.00690496	0.52970577	AT4G27290.:	BP	maker-scaffold_10-augustus-gene-42.45-	1.32046452
Insect	epiGBS	GO:0009057	macromolecule catabolic process	0.00695918	0.00695918	0.52970577	AT5G06460.:	BP	maker-scaffold_9-snap-gene-24.107-mRN	1.36874876
Insect	epiGBS	GO:0071103	DNA conformation change	0.00752334	0.00752334	0.53183513	AT1G79950.:	BP	maker-scaffold_6-snap-gene-59.48-mRNA	2.02177933
Insect	epiGBS	GO:0051241	negative regulation of multicellular orga	0.00796144	0.00796144	0.53183513	AT1G30970.:	BP	snap_masked-scaffold_6-processed-gene-	2.00898326
Insect	epiGBS	GO:1901361	organic cyclic compound catabolic proce	0.00796697	0.00796697	0.53183513	AT5G54080.:	BP	maker-scaffold_10-augustus-gene-20.65-	1.66221616
Insect	epiGBS	GO:0044265	cellular macromolecule catabolic proces	0.00817814	0.00817814	0.53183513	AT5G06460.:	BP	maker-scaffold_9-snap-gene-24.107-mRN	1.40193548
Insect	epiGBS	GO:0000302	response to reactive oxygen species	0.00888341	0.00888341	0.53183513	AT2G28190.:	BP	maker-scaffold_7-augustus-gene-2.65-mR	1.93474654
Insect	epiGBS	GO:0007062	sister chromatid cohesion	0.00924973	0.00924973	0.53183513	AT5G47690.:	BP	maker-scaffold_4-snap-gene-69.82-mRNA	3.77880184
Insect	epiGBS	GO:0034599	cellular response to oxidative stress	0.00989778	0.00989778	0.53183513	AT2G28190.:	BP	maker-scaffold_7-augustus-gene-2.65-mR	2.35125448
Insect	epiGBS	GO:0090305	nucleic acid phosphodiester bond hydrol	0.01015813	0.01015813	0.53183513	AT1G55870.:	BP	maker-scaffold_10-augustus-gene-17.55-	1.76344086
Insect	epiGBS	GO:1901565	organonitrogen compound catabolic pro	0.01045869	0.01045869	0.53183513	AT5G54080.:	BP	maker-scaffold_10-augustus-gene-20.65-	1.37410976
Insect	epiGBS	GO:0051568	histone H3-K4 methylation	0.01075148	0.01075148	0.53183513	AT4G15180.:	BP	maker-scaffold_51-augustus-gene-2.41-m	3.64849833
Insect	epiGBS	GO:0048268	clathrin coat assembly	0.01087145	0.01087145	0.53183513	AT2G40060.:	BP	maker-scaffold_11-augustus-gene-20.76-	4.45500849
Insect	epiGBS	GO:0006004	fucose metabolic process	0.01190222	0.01190222	0.53183513	AT1G76270.:	BP	maker-scaffold_7-augustus-gene-52.110-	2.7948874
Insect	epiGBS	GO:0051093	negative regulation of developmental pr	0.01232058	0.01232058	0.53183513	AT1G30970.:	BP	snap_masked-scaffold_6-processed-gene-	1.8261012
Insect	epiGBS	GO:0048581	negative regulation of post-embryonic d	0.01299059	0.01299059	0.53183513	AT1G30970.:	BP	snap_masked-scaffold_6-processed-gene-	2.00800565
Insect	epiGBS	GO:0000303	response to superoxide	0.01309081	0.01309081	0.53183513	AT2G28190.:	BP	maker-scaffold_7-augustus-gene-2.65-mR	4.23225807
Insect	epiGBS	GO:0000305	response to oxygen radical	0.01309081	0.01309081	0.53183513	AT2G28190.:	BP	maker-scaffold_7-augustus-gene-2.65-mR	4.23225807
Insect	epiGBS	GO:0016075	rRNA catabolic process	0.01309081	0.01309081	0.53183513	AT3G60500.:	BP	maker-scaffold_7-snap-gene-23.104-mRN	4.23225807
Insect	epiGBS	GO:0045995	regulation of embryonic development	0.01309081	0.01309081	0.53183513	AT2G19450.:	BP	maker-scaffold_13-snap-gene-33.63-mRN	4.23225807
Insect	epiGBS	GO:0009112	nucleobase metabolic process	0.01341449	0.01341449	0.53183513	AT5G23300.:	BP	snap_masked-scaffold_28-processed-gen	3.02304148
Insect	epiGBS	GO:0033866	nucleoside bisphosphate biosynthetic pr	0.01341449	0.01341449	0.53183513	AT1G09430.:	BP	maker-scaffold_44-snap-gene-9.114-mRN	3.02304148
Insect	epiGBS	GO:0034030	ribonucleoside bisphosphate biosyntheti	0.01341449	0.01341449	0.53183513	AT1G09430.:	BP	maker-scaffold_44-snap-gene-9.114-mRN	3.02304148

stress	technique	ID	Description	pvalue	p.adjust	qvalue	geneID	bgsource	PN_annoated_genes	foldEnrichme
Artificial	epiGBS	GO:0006396	RNA processing	3.97E-06	3.97E-06	0.00443481	AT1G55870.	BP	maker-scaffold_10-augustus-gene-17.55-	1.62003774
Artificial	epiGBS	GO:0006397	mRNA processing	5.84E-06	5.84E-06	0.00443481	AT1G55870.	BP	maker-scaffold_10-augustus-gene-17.55-	1.96426245
Artificial	epiGBS	GO:0006996	organelle organization	9.27E-05	9.27E-05	0.05189426	AT5G55310.	BP	maker-scaffold_10-augustus-gene-13.32-	1.3323111
Artificial	epiGBS	GO:0045292	mRNA cis splicing, via spliceosome	0.00011386	0.00011386	0.05189426	AT1G44910.	BP	maker-scaffold_9-snap-gene-28.97-mRNA	5.77206704
Artificial	epiGBS	GO:0016071	mRNA metabolic process	0.00021484	0.00021484	0.08160089	AT1G55870.	BP	maker-scaffold_10-augustus-gene-17.55-	1.64611365
Artificial	epiGBS	GO:0000394	RNA splicing, via endonucleolytic cleava	0.00033974	0.00033974	0.09811535	AT1G07910.	BP	augustus_masked-scaffold_17-processed-	5.8205718
Artificial	epiGBS	GO:0000398	mRNA splicing, via spliceosome	0.00034593	0.00034593	0.09811535	AT1G44910.	BP	maker-scaffold_9-snap-gene-28.97-mRNA	2.10352297
Artificial	epiGBS	GO:0051641	cellular localization	0.00038748	0.00038748	0.09811535	AT5G54750.	BP	maker-scaffold_10-augustus-gene-47.66-	1.4161779
Artificial	epiGBS	GO:2001020	regulation of response to DNA damage s	0.00050654	0.00050654	0.10354117	AT3G48710.	BP	maker-scaffold_19-augustus-gene-5.104-	4.12290503
Artificial	epiGBS	GO:0010506	regulation of autophagy	0.00054051	0.00054051	0.10354117	AT1G50030.	BP	maker-scaffold_9-snap-gene-28.92-mRNA	4.61765363
Artificial	epiGBS	GO:0051649	establishment of localization in cell	0.00054521	0.00054521	0.10354117	AT5G54750.	BP	maker-scaffold_10-augustus-gene-47.66-	1.46079619
Artificial	epiGBS	GO:0046907	intracellular transport	0.00066495	0.00066495	0.10572249	AT5G54750.	BP	maker-scaffold_10-augustus-gene-47.66-	1.46773381
Artificial	epiGBS	GO:0006388	tRNA splicing, via endonucleolytic cleava	0.00069355	0.00069355	0.10572249	AT1G07910.	BP	augustus_masked-scaffold_17-processed-	6.34293081
Artificial	epiGBS	GO:0051276	chromosome organization	0.00069586	0.00069586	0.10572249	AT5G55310.	BP	maker-scaffold_10-augustus-gene-13.32-	1.54028014
Artificial	epiGBS	GO:0006259	DNA metabolic process	0.00077979	0.00077979	0.11106887	AT5G55310.	BP	maker-scaffold_10-augustus-gene-13.32-	1.60893855
Artificial	epiGBS	GO:0033043	regulation of organelle organization	0.00093533	0.00093533	0.1196026	AT3G27120.	BP	maker-scaffold_9-augustus-gene-47.67-m	1.91104611
Artificial	epiGBS	GO:0018107	peptidyl-threonine phosphorylation	0.00102524	0.00102524	0.1196026	AT2G23070.	BP	maker-scaffold_33-augustus-gene-11.24-	5.88986433
Artificial	epiGBS	GO:0018210	peptidyl-threonine modification	0.00102524	0.00102524	0.1196026	AT2G23070.	BP	maker-scaffold_33-augustus-gene-11.24-	5.88986433
Artificial	epiGBS	GO:0051128	regulation of cellular component organi	0.00104963	0.00104963	0.1196026	AT3G27120.	BP	maker-scaffold_9-augustus-gene-47.67-m	1.64524476
Artificial	epiGBS	GO:0006352	DNA-templated transcription, initiation	0.0011133	0.0011133	0.1208165	AT1G54140.	BP	maker-scaffold_6-augustus-gene-52.52-m	2.64680323
Artificial	epiGBS	GO:0034614	cellular response to reactive oxygen spe	0.00131028	0.00131028	0.12739196	AT2G28190.	BP	maker-scaffold_7-augustus-gene-2.65-mR	3.29832402
Artificial	epiGBS	GO:0030641	regulation of cellular pH	0.00141871	0.00141871	0.12739196	AT5G62670.	BP	maker-scaffold_21-augustus-gene-3.67-m	3.98073589
Artificial	epiGBS	GO:0051453	regulation of intracellular pH	0.00141871	0.00141871	0.12739196	AT5G62670.	BP	maker-scaffold_21-augustus-gene-3.67-m	3.98073589
Artificial	epiGBS	GO:0000375	RNA splicing, via transesterification rea	0.00145339	0.00145339	0.12739196	AT1G44910.	BP	maker-scaffold_9-snap-gene-28.97-mRNA	1.88062335
Artificial	epiGBS	GO:0000377	RNA splicing, via transesterification rea	0.00145339	0.00145339	0.12739196	AT1G44910.	BP	maker-scaffold_9-snap-gene-28.97-mRNA	1.88062335
Artificial	epiGBS	GO:0098659	inorganic cation import across plasma m	0.00175491	0.00175491	0.14757115	AT2G26650.	BP	maker-scaffold_4-augustus-gene-4.36-mR	3.84804469
Artificial	epiGBS	GO:0033554	cellular response to stress	0.00181311	0.00181311	0.14757115	AT1G53210.	BP	maker-scaffold_10-augustus-gene-21.41-	1.32184741
Artificial	epiGBS	GO:0006281	DNA repair	0.0023922	0.0023922	0.18607457	AT3G27120.	BP	maker-scaffold_9-augustus-gene-47.67-m	1.66879489
Artificial	epiGBS	GO:0031503	protein-containing complex localization	0.00245508	0.00245508	0.18607457	AT5G16790.	BP	maker-scaffold_24-augustus-gene-20.60-	2.66776208
Artificial	epiGBS	GO:0090333	regulation of stomatal closure	0.00267511	0.00267511	0.18607457	AT1G47128.	BP	maker-scaffold_9-snap-gene-33.131-mRN	2.79518985
Artificial	epiGBS	GO:0043484	regulation of RNA splicing	0.00314203	0.00314203	0.18607457	AT5G62600.	BP	maker-scaffold_21-augustus-gene-4.64-m	3.49822245
Artificial	epiGBS	GO:0099587	inorganic ion import across plasma mem	0.00314203	0.00314203	0.18607457	AT2G26650.	BP	maker-scaffold_4-augustus-gene-4.36-mR	3.49822245
Artificial	epiGBS	GO:0006904	vesicle docking involved in exocytosis	0.00314434	0.00314434	0.18607457	AT1G02010.	BP	maker-scaffold_3-augustus-gene-24.104-	5.99695277
Artificial	epiGBS	GO:0070301	cellular response to hydrogen peroxide	0.00314434	0.00314434	0.18607457	AT3G59410.	BP	maker-scaffold_14-augustus-gene-32.79-	5.99695277
Artificial	epiGBS	GO:0006282	regulation of DNA repair	0.00320304	0.00320304	0.18607457	AT3G48710.	BP	maker-scaffold_19-augustus-gene-5.104-	3.95798883
Artificial	epiGBS	GO:0006885	regulation of pH	0.00331934	0.00331934	0.18607457	AT5G62670.	BP	maker-scaffold_21-augustus-gene-3.67-m	3.14126097
Artificial	epiGBS	GO:0008064	regulation of actin polymerization or dep	0.00331934	0.00331934	0.18607457	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	3.14126097
Artificial	epiGBS	GO:0030832	regulation of actin filament length	0.00331934	0.00331934	0.18607457	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	3.14126097
Artificial	epiGBS	GO:0110053	regulation of actin filament organizatior	0.00331934	0.00331934	0.18607457	AT2G41740.	BP	maker-scaffold_17-snap-gene-14.101-mR	3.14126097
Artificial	epiGBS	GO:0006974	cellular response to DNA damage stimul	0.00334762	0.00334762	0.18607457	AT3G27120.	BP	maker-scaffold_9-augustus-gene-47.67-m	1.60893855
Artificial	epiGBS	GO:0071166	ribonucleoprotein complex localization	0.00372116	0.00372116	0.19478239	AT5G16790.	BP	maker-scaffold_24-augustus-gene-20.60-	2.85431887
Artificial	epiGBS	GO:0098876	vesicle-mediated transport to the plasm	0.00386554	0.00386554	0.19478239	AT2G27900.	BP	maker-scaffold_7-snap-gene-1.74-mRNA-	3.06820839
Artificial	epiGBS	GO:0051052	regulation of DNA metabolic process	0.00388358	0.00388358	0.19478239	AT3G27120.	BP	maker-scaffold_9-augustus-gene-47.67-m	2.51955307
Artificial	epiGBS	GO:1990573	potassium ion import across plasma me	0.00395356	0.00395356	0.19478239	AT2G26650.	BP	maker-scaffold_4-augustus-gene-4.36-mR	3.80575849
Artificial	epiGBS	GO:0006913	nucleocytoplasmic transport	0.00401711	0.00401711	0.19478239	AT5G16790.	BP	maker-scaffold_24-augustus-gene-20.60-	2.15108088
Artificial	epiGBS	GO:0051169	nuclear transport	0.00401711	0.00401711	0.19478239	AT5G16790.	BP	maker-scaffold_24-augustus-gene-20.60-	2.15108088
Artificial	epiGBS	GO:0007030	Golgi organization	0.00447858	0.00447858	0.21263439	AT4G11150.	BP	maker-scaffold_24-augustus-gene-5.51-m	2.99847638
Artificial	epiGBS	GO:0051130	positive regulation of cellular componen	0.00473083	0.00473083	0.22002679	AT3G52140.	BP	maker-scaffold_7-augustus-gene-29.88-m	2.11431027
Artificial	epiGBS	GO:0031123	RNA 3'-end processing	0.00490909	0.00490909	0.22375096	AT5G14580.	BP	snap_masked-scaffold_10-processed-gen	2.57681564
Artificial	epiGBS	GO:0051168	nuclear export	0.00549413	0.00549413	0.24550668	AT5G16790.	BP	maker-scaffold_24-augustus-gene-20.60-	2.53717233

stress	technique	ID	Description	pvalue	p.adjust	qvalue	geneID	bgsource	PN_annoated_genes	foldEnrichme
Insect	epiGBS	GO:0004519	endonuclease activity	0.0423319	0.0423319	0.46947029	AT1G19100.	MF	maker-scaffold_21-snap-gene-12.84-mRN	1.86509549
Insect	epiGBS	GO:0050660	flavin adenine dinucleotide binding	0.04341839	0.04341839	0.46955836	AT3G23410.	MF	maker-scaffold_25-snap-gene-9.98-mRNA	1.70019375
Insect	epiGBS	GO:0005451	monovalent cation:proton antiporter act	0.04455682	0.04455682	0.46955836	AT2G01980.	MF	maker-scaffold_11-snap-gene-46.176-mR	2.55340454
Insect	epiGBS	GO:0015298	solute:cation antiporter activity	0.04536411	0.04536411	0.46955836	AT2G01980.	MF	maker-scaffold_11-snap-gene-46.176-mR	2.29806409
Insect	epiGBS	GO:0030276	clathrin binding	0.04536411	0.04536411	0.46955836	AT2G40060.	MF	maker-scaffold_11-augustus-gene-20.76-	2.29806409
Insect	epiGBS	GO:0005319	lipid transporter activity	0.04705261	0.04705261	0.47905156	AT4G39850.	MF	maker-scaffold_14-augustus-gene-19.66-	2.11465052
Insect	epiGBS	GO:0015299	solute:proton antiporter activity	0.04856407	0.04856407	0.48170099	AT2G01980.	MF	maker-scaffold_11-snap-gene-46.176-mR	2.49402304
Insect	epiGBS	GO:0015385	sodium:proton antiporter activity	0.04886408	0.04886408	0.48170099	AT2G01980.	MF	maker-scaffold_11-snap-gene-46.176-mR	3.57476636
Insect	epiGBS	GO:0000151	ubiquitin ligase complex	0.00094746	0.00094746	0.10908914	AT3G17205.	CC	maker-scaffold_11-augustus-gene-31.72-	1.86946567
Insect	epiGBS	GO:0031974	membrane-enclosed lumen	0.00121923	0.00121923	0.10908914	AT2G44350.	CC	augustus_masked-scaffold_9-processed-g	1.40649455
Insect	epiGBS	GO:0043233	organelle lumen	0.00121923	0.00121923	0.10908914	AT2G44350.	CC	augustus_masked-scaffold_9-processed-g	1.40649455
Insect	epiGBS	GO:0070013	intracellular organelle lumen	0.00121923	0.00121923	0.10908914	AT2G44350.	CC	augustus_masked-scaffold_9-processed-g	1.40649455
Insect	epiGBS	GO:0030660	Golgi-associated vesicle membrane	0.00237316	0.00237316	0.14710431	AT4G33410.	CC	augustus_masked-scaffold_21-processed-	3.72281527
Insect	epiGBS	GO:0000177	cytoplasmic exosome (RNase complex)	0.00246616	0.00246616	0.14710431	AT3G60500.	CC	maker-scaffold_7-snap-gene-23.104-mRN	6.54560926
Insect	epiGBS	GO:0031461	cullin-RING ubiquitin ligase complex	0.00389447	0.00389447	0.1864074	AT2G34260.	CC	augustus_masked-scaffold_43-processed-	1.83252544
Insect	epiGBS	GO:0005798	Golgi-associated vesicle	0.00443226	0.00443226	0.1864074	AT4G33410.	CC	augustus_masked-scaffold_21-processed-	3.03903287
Insect	epiGBS	GO:0031981	nuclear lumen	0.00493989	0.00493989	0.1864074	AT1G72390.	CC	maker-scaffold_6-snap-gene-51.77-mRNA	1.38785599
Insect	epiGBS	GO:1990234	transferase complex	0.00626504	0.00626504	0.1864074	AT5G60040.	CC	maker-scaffold_10-snap-gene-1.54-mRNA	1.4457535
Insect	epiGBS	GO:0097708	intracellular vesicle	0.00700486	0.00700486	0.1864074	AT3G16200.	CC	snap_masked-scaffold_6-processed-gene	1.36558348
Insect	epiGBS	GO:0012510	trans-Golgi network transport vesicle m	0.00706573	0.00706573	0.1864074	AT2G40060.	CC	maker-scaffold_11-augustus-gene-20.76-	5.0054659
Insect	epiGBS	GO:0005764	lysosome	0.0074847	0.0074847	0.1864074	AT5G64500.	CC	maker-scaffold_7-augustus-gene-34.102-	2.78993182
Insect	epiGBS	GO:0030140	trans-Golgi network transport vesicle	0.00773266	0.00773266	0.1864074	AT2G40060.	CC	maker-scaffold_11-augustus-gene-20.76-	3.93948705
Insect	epiGBS	GO:0031982	vesicle	0.00781266	0.00781266	0.1864074	AT3G16200.	CC	snap_masked-scaffold_6-processed-gene	1.35133127
Insect	epiGBS	GO:0031410	cytoplasmic vesicle	0.00965863	0.00965863	0.20401101	AT3G16200.	CC	snap_masked-scaffold_6-processed-gene	1.34830332
Insect	epiGBS	GO:0034518	RNA cap binding complex	0.00969052	0.00969052	0.20401101	AT2G27100.	CC	maker-scaffold_16-augustus-gene-12.88-	6.38196903
Insect	epiGBS	GO:0016604	nuclear body	0.01114146	0.01114146	0.22152611	AT1G72390.	CC	maker-scaffold_6-snap-gene-51.77-mRNA	2.04853327
Insect	epiGBS	GO:0005732	small nucleolar ribonucleoprotein compl	0.01286267	0.01286267	0.2247557	AT5G52470.	CC	maker-scaffold_25-snap-gene-18.70-mRN	4.25464602
Insect	epiGBS	GO:0000323	lytic vacuole	0.0129745	0.0129745	0.2247557	AT5G64500.	CC	maker-scaffold_7-augustus-gene-34.102-	2.54008718
Insect	epiGBS	GO:0080008	Cul4-RING E3 ubiquitin ligase complex	0.01318787	0.01318787	0.2247557	AT2G34260.	CC	augustus_masked-scaffold_43-processed-	1.94657007
Insect	epiGBS	GO:0009574	preprophase band	0.01394761	0.01394761	0.22689899	AT2G34680.	CC	maker-scaffold_10-augustus-gene-11.37-	3.43116614
Insect	epiGBS	GO:0016607	nuclear speck	0.01470588	0.01470588	0.22830991	AT1G24706.	CC	maker-scaffold_24-snap-gene-23.45-mRN	2.21596147
Insect	epiGBS	GO:0000178	exosome (RNase complex)	0.01531019	0.01531019	0.22830991	AT3G60500.	CC	maker-scaffold_7-snap-gene-23.104-mRN	4.05204383
Insect	epiGBS	GO:1905354	exoribonuclease complex	0.01803186	0.01803186	0.25814031	AT3G60500.	CC	maker-scaffold_7-snap-gene-23.104-mRN	3.86786002
Insect	epiGBS	GO:1902494	catalytic complex	0.01999372	0.01999372	0.25960461	AT5G19130.	CC	augustus_masked-scaffold_10-processed-	1.27427002
Insect	epiGBS	GO:0005905	clathrin-coated pit	0.02035701	0.02035701	0.25960461	AT2G40060.	CC	maker-scaffold_11-augustus-gene-20.76-	3.12841619
Insect	epiGBS	GO:0005849	mRNA cleavage factor complex	0.02078988	0.02078988	0.25960461	AT4G33410.	CC	augustus_masked-scaffold_21-processed-	4.90920694
Insect	epiGBS	GO:0005686	U2 snRNP	0.02103561	0.02103561	0.25960461	AT5G16260.	CC	snap_masked-scaffold_5-processed-gene	3.69969219
Insect	epiGBS	GO:0000176	nuclear exosome (RNase complex)	0.02555296	0.02555296	0.29500873	AT3G60500.	CC	maker-scaffold_7-snap-gene-23.104-mRN	4.55854931
Insect	epiGBS	GO:0030132	clathrin coat of coated pit	0.02555296	0.02555296	0.29500873	AT2G40060.	CC	maker-scaffold_11-augustus-gene-20.76-	4.55854931
Insect	epiGBS	GO:0030130	clathrin coat of trans-Golgi network vesi	0.03084935	0.03084935	0.32473005	AT2G40060.	CC	maker-scaffold_11-augustus-gene-20.76-	4.25464602
Insect	epiGBS	GO:0071458	integral component of cytoplasmic side	0.03084935	0.03084935	0.32473005	AT4G33410.	CC	augustus_masked-scaffold_21-processed-	4.25464602
Insect	epiGBS	GO:0098554	cytoplasmic side of endoplasmic reticulu	0.03084935	0.03084935	0.32473005	AT4G33410.	CC	augustus_masked-scaffold_21-processed-	4.25464602
Insect	epiGBS	GO:0036452	ESCRT complex	0.03179914	0.03179914	0.3251641	AT2G06530.	CC	maker-scaffold_6-augustus-gene-54.60-m	3.27280463
Insect	epiGBS	GO:0032040	small-subunit processome	0.03435878	0.03435878	0.33667483	AT5G16750.	CC	augustus_masked-scaffold_24-processed-	2.45460347
Insect	epiGBS	GO:0030665	clathrin-coated vesicle membrane	0.03480624	0.03480624	0.33667483	AT2G40060.	CC	maker-scaffold_11-augustus-gene-20.76-	2.72733719
Insect	epiGBS	GO:0005654	nucleoplasm	0.04050784	0.04050784	0.38151425	AT1G72390.	CC	maker-scaffold_6-snap-gene-51.77-mRNA	1.41821534
Insect	epiGBS	GO:1990391	DNA repair complex	0.04301867	0.04301867	0.39477322	AT3G18524.	CC	maker-scaffold_31-augustus-gene-3.35-m	3.75409943
Insect	epiGBS	GO:0030136	clathrin-coated vesicle	0.04623557	0.04623557	0.40876119	AT2G40060.	CC	maker-scaffold_11-augustus-gene-20.76-	2.00218636
Insect	epiGBS	GO:0009524	phragmoplast	0.0468272	0.0468272	0.40876119	AT2G34680.	CC	maker-scaffold_10-augustus-gene-11.37-	1.77276917
Artificial	epiGBS	GO:0008380	RNA splicing	1.00E-07	1.00E-07	0.00022899	AT1G44910.	BP	maker-scaffold_9-snap-gene-28.97-mRNA	2.35594573

Appendix A

Technical analysis of global methylation with fragments shared by WGBS and epiGBS

Methods

We searched for the common fragments obtained in epiGBS-R and WGBS (Table A1). Only those fragments with substantial methylation information: ≥ 5 cytosines for CpG or CHG, and ≥10 cytosines for CHH were retained and their average methylation level calculated. Linear models were applied to fragment methylation data to test for the effects of herbivory treatment (with three levels), ortet (with three levels), and technique (epiGBS-R vs WGBS) as fixed factors. Significance of fixed factors and their interaction was tested using function ANOVA (package car, v3.0-12.) (Fox et al.,2019) The frequency of the average methylation percentage of shared fragments for each herbivory among ortets was compared between two methods by Pearson's Chi-square test, using chisq.test function of stats R package v3.6.2).

Finally, principal components analyses were run to detect multivariate patterns of DNA methylation variation among ramets assigned to different herbivory treatments and the effect of technique applied. The analysis was conducted on average methylation of the shared fragments obtained by epiGBS-R and WGBS, using the correlation matrix and the built-in R function "prcomp" of the stats package with default parameters.

Table A1. Total number of common methylated positions captured by epiGBS-R (reference branch) and WGBS in the three sequence contexts (CpG, CHG and CHH) with and without missing values (With NAs and Without NAs). Values were obtained using methylkit and taking into account positions with methylation information of ≥5 cytosines for CpG or CHG, and ≥10 cytosines for CHH in WGBS; and ≥10 cytosines for all contexts in epiGBS-R.

Technique	Context	With NAs	Without NAs	
		#	#	%
epiGBS-R	CpG	28,826	2,243	7.78
	CHG	30,954	3,771	12.18
	CHH	35,375	6,781	19.17
WGBS	CpG	32,459	1,569	4.83
	CHG	35,209	3,246	9.22
	CHH	38,907	7,222	18.56

Results

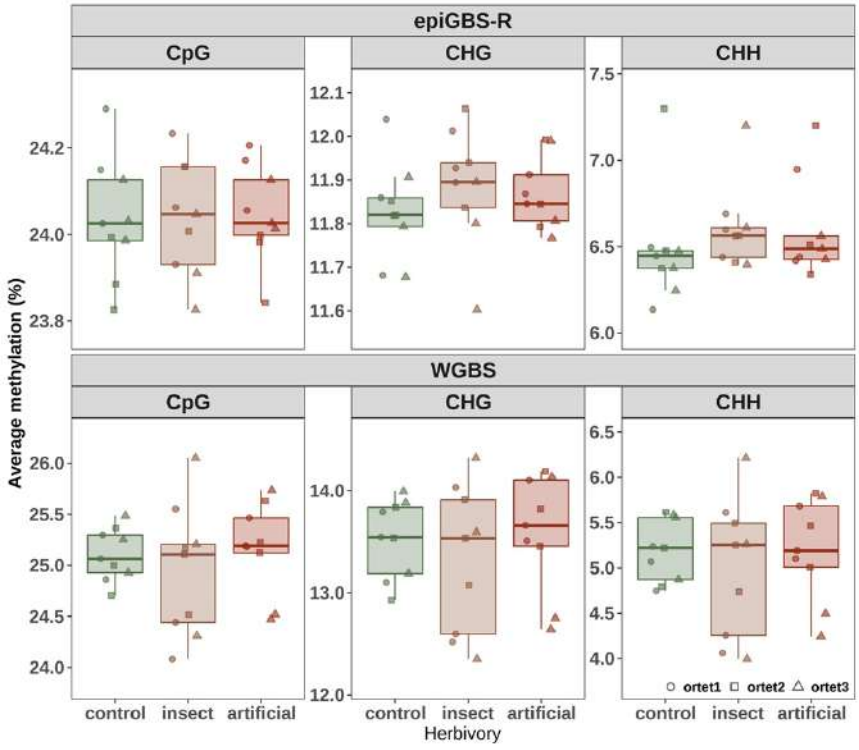
For the subset of shared fragments, the estimates of global cytosine methylation level in the leaf genomes of the poplar ramets studied ranged from 3.9% to 26.1% according to WGBS and from 6.1% to 24.2% according epiGBS-R. The ANOVA tests detected that methylation levels obtained by the two techniques were significantly different in the three contexts (A1 Fig; $P < 0.005$) for these common fragments. Average methylation was relatively higher in WGBS for CpG and CHG, whereas CHH methylation percentage was higher in epiGBS-R (A1 Fig), differences being statistically significant only in CHG and CHH contexts (Table A2). Furthermore, global methylation in leaf genomes did not differ between ortets, or the three herbivory levels, or the interaction between the study factors was never statistically different but were always significantly different in the three cytosine sequence contexts between the techniques (Table A2).

Table A2. Summary of the ANOVA results carried out to test the effect of ortet, herbivory treatment, technique and their interaction on genome wide DNA methylation for each of the three cytosine contexts performed with the common fragments of epiGBS-R and WGBS techniques.

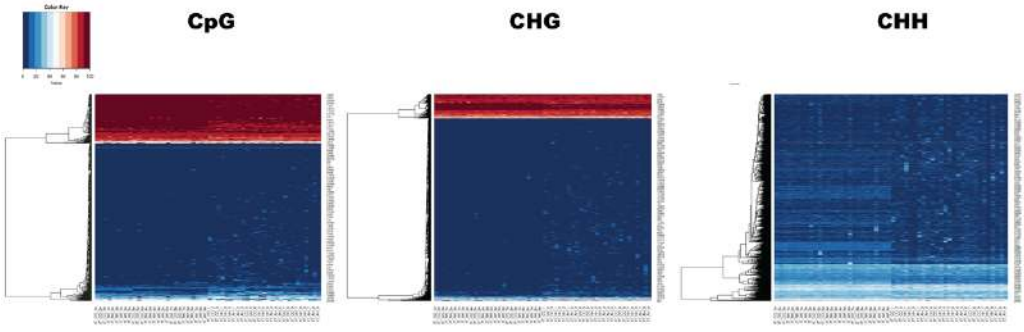
Context	Source of variation	d.f.	F	p
CpG	ortet	2	0.03	0.97
	herbivory	2	0.53	0.61
	technique	1	107.36	**<0.0001**
	ortet × herbivory	4	0.67	0.61
	ortet ×technique	2	0.51	0.60
	ortet × herbivory × technique	4	0.84	0.50
CHG	ortet	2	0.34	0.71
	herbivory	2	0.35	0.70
	technique	1	185.08	**<0.0001**
	ortet × herbivory	4	0.61	0.65
	ortet × technique	2	0.16	0.85
	ortet × herbivory × technique	4	0.82	0.52
CHH	ortet	2	0.78	0.46

herbivory	2	0.19	0.82
technique	1	117.63	**<0.0001**
ortet × herbivory	4	0.56	0.56
ortet × technique	2	0.90	0.89
ortet × herbivory × technique	4	0.69	0.69

Heatmap representation (A2 Fig) of average fragment methylation showed congruency between the two techniques for all three contexts across all 27 ramets (x-axis) and scaffolds (y-axis) and a large difference in the patterns observed for each context. Fragments had frequently very high or very low methylation in CpG and CHG contexts, with a lack of cases with intermediate methylation in those two contexts, whereas in CHH average methylation per fragment varies between low and intermediate but almost never reach a methylation > 50 %.

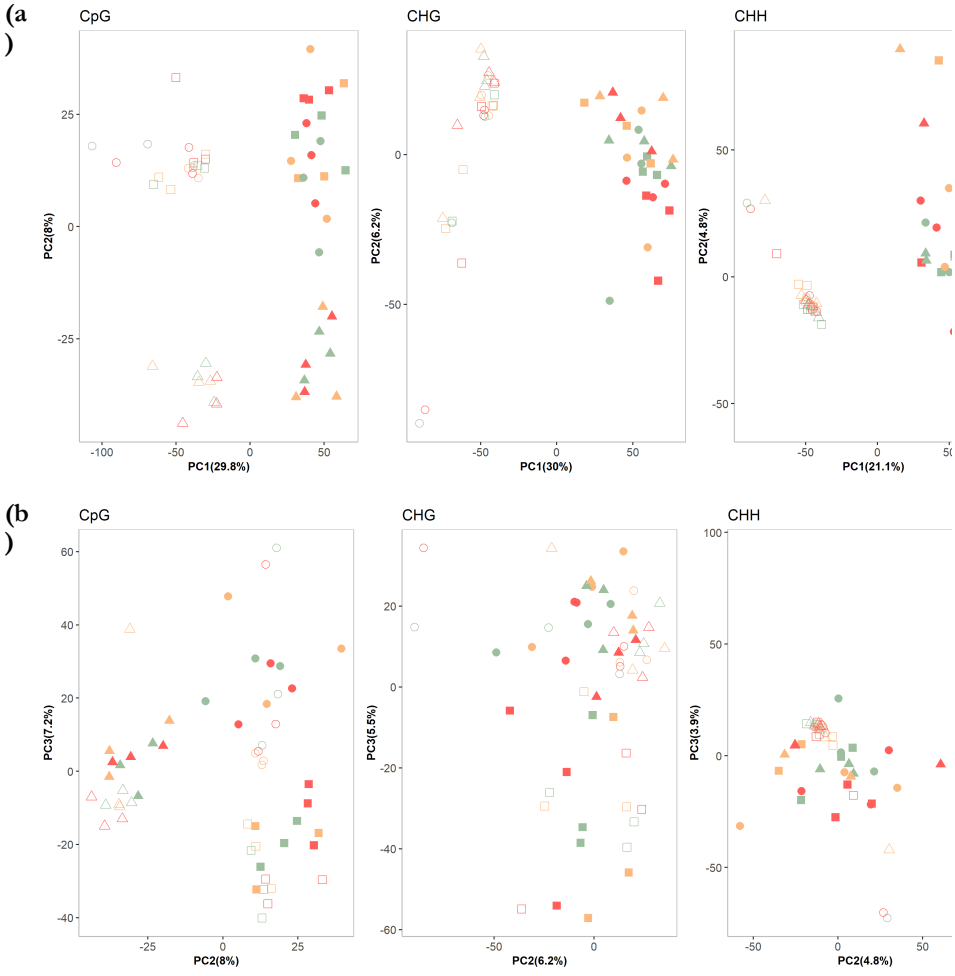


A1 Fig: Average methylation percentage estimated from common fragments captured by epiGBS-R and WGBS. (a) Boxplots of the average global DNA methylation level (%) obtained for controls, insect and artificial herbivory treated plants in each context (CpG, CHG and CHH) obtained by epiGBS-R and WGBS techniques. Treatments are shown in different colors (control: green, insect herbivory: brown, and artificial herbivory: red).



A2 Fig: Heatmap visualization of per-fragment average methylation based on common fragments in the three sequence contexts for each of the 27 samples that are shown ordered in the x-axis (left: epiGBS-R, right: WGBS). Hierarchical clustering (Ward's method) was performed on the fragments' methylation estimate (Manhattan distance). A scale is shown on the right, in which blue and red correspond to a lower and a higher methylation status, respectively.

As expected, principal component analysis showed that the primary source of variation in average methylation per fragment (PC1) was associated with the technique applied in all three contexts (29.8%, 30% and 21.1% for CG, CHG and CHH, respectively). Samples from each technique clustered together more closely along the PC1 principal component (A3 Fig). The second and third components (PC2 and PC3) explained only between 3.9% and 8.0 % of the variance in average methylation per fragment for the three different contexts. In CpG and CHG contexts, samples from each of the three study ortets clustered separately along the PC2 and PC3 axes, indicating that fragment methylation patterns differ between ortets, with ortet1 being the most distinct in both CpG and CHG contexts. However, in CHH context, PC2 and PC3 did not reveal any differences between ortets, treatments or techniques, and a larger variation among WGBS samples was observed when compared to variation among epiGBS-R samples.



A3 Fig: PCA ordination plots on the DNA methylation profiles of the common loci captured by both epiGBS-R and WGBS in CpG, CHG and CHH context. (a) PC1 versus PC2. (b) PC2 versus PC3. Ortets are shown in different shapes and herbivory treatments are represented with different colors (control: green, insect: yellow, artificial: red). Filled and empty shapes represent WGBS and epiGBS samples, respectively.

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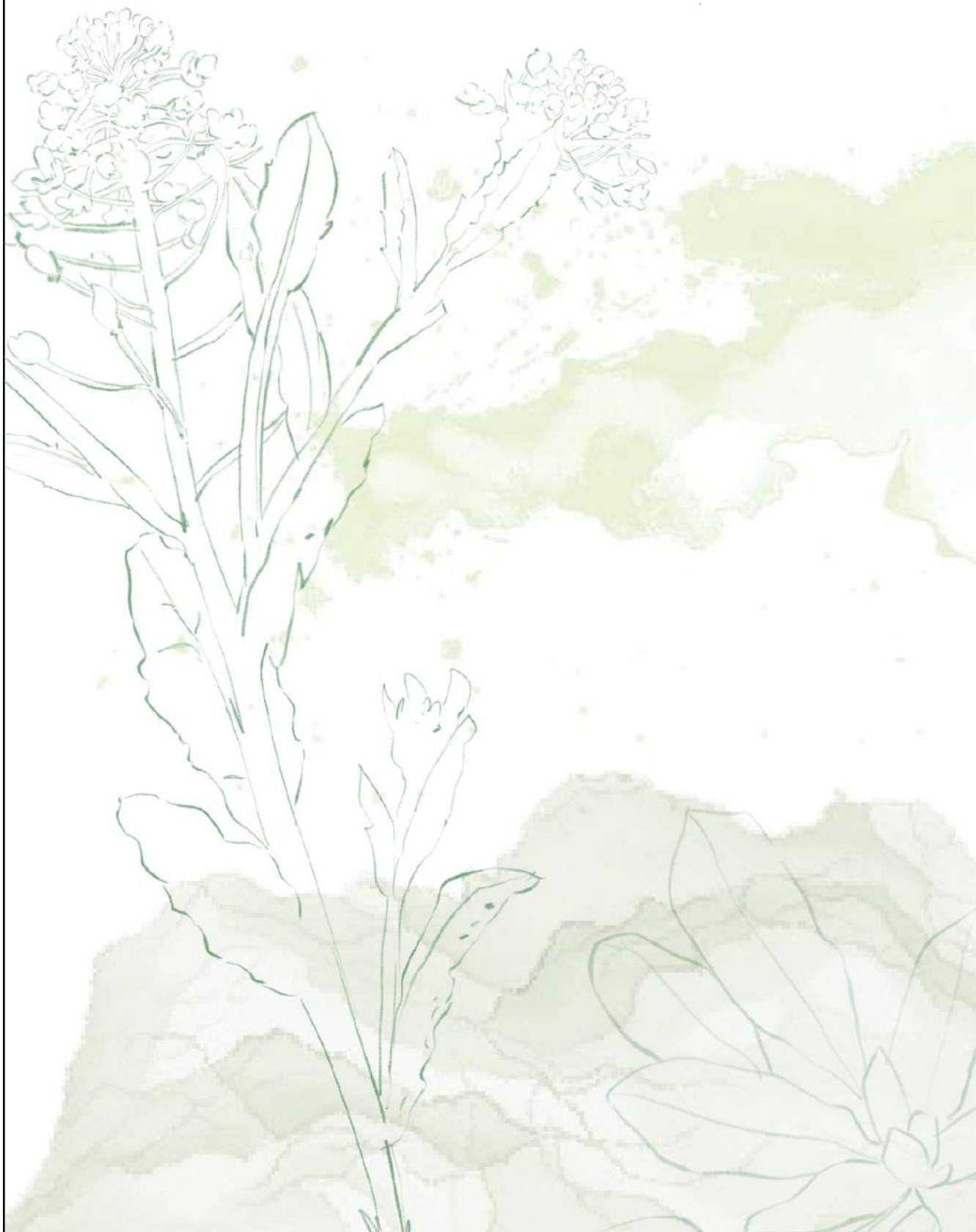
GENERAL DISCUSSION



GENERAL DISCUSSION

As sessile organisms, plants are constantly exposed to a wide range of environmental fluctuations in abiotic conditions (e.g., temperature, drought, precipitation, nutrients) and biotic interactions (e.g., herbivores, pathogens, mycorrhiza) that can have effect on their growth, reproduction and survival. When fluctuation in abiotic or biotic factors become extreme, plants experience stress and their response would vary according to life-history traits (e.g., lifespan, individual size, reproductive mode, and other demographic traits), physiological traits (e.g., photosynthetic capacity, stomatal conductance) and the environmental context (Mosa et al., 2017). This PhD Thesis addresses the role of epigenetic regulation in modulating plant biotic interactions (Alonso et al., 2019; Ramos-Cruz et al., 2021), a topic that remains understudied in comparison with the much more frequently addressed plant epigenetic response to osmotic and heat stress, heavy metals contamination or light and resource availability (see e.g., Alonso et al., 2016 and references therein). As discussed in **Chapter 1**, several epigenetic mechanisms including DNA cytosine methylation, histone modification and small non-coding RNA production might be relevant in this ecological context because they function in combination rather than independently in regulating gene expression, genome stability and phenotypic plasticity (Liu & Chang, 2021; Talbert et al., 2019; J. Wang et al., 2016).

The Thesis was developed within the *EpiDiverse* Marie-Curie Innovative Training Network that contributed to generate improved genomic resources (see e.g., Dubay 2024 for *Populus nigra* 'italica'; Nunn et al., 2022 for *Thlaspi arvense*) and new bioinformatic tools (see e.g., Nunn et al., 2021; Gawehns et al., 2022) that were used within this Thesis. This consortium, that

joined with 12 research groups from ecology, molecular (epi)genetics and bioinformatics, also provided new insights into the ecological significance of epigenetic variation in non-model plant species (see e.g.,Galanti et al., 2022; Díez Rodríguez et al., 2022a, Troyee et al., 2023; Van Antro et al., 2023; Sammarco et al., 2024).This has contributed to fill the gap between deep molecular understanding, traditionally reserved for a few model plant species, and the broader ecological perspective required to better understand the complex relationships among genetics, epigenetics, phenotype and environment in natural plant populations (Richards et al., 2017).

This Thesis investigates the general hypothesis that herbivory can cause epigenetic modifications in plants, and these will contribute to activate plant defense mechanisms. To test it, we analyzed DNA methylation variations in response to leaf consumption by Lepidopteran larvae (i.e., insect herbivory) and manual defoliation together with jasmonic acid application (i.e., artificial herbivory), in individuals collected from geographically distant European populations of two species with contrasting features: the ruderal annual pennycress, *Thlaspi arvense* (Brassicaceae), and the Lombardy poplar, *Populus nigra* 'italica' (Salicaceae), a clonally propagated variety of this fast growing deciduous tree. As the secondary compounds present on each plant species conditioned the insect herbivores that could be used, we selected larvae of *Pieris brassicae* and *Lymantria dispar* for *T. arvense* and *P. nigra*, respectively. The artificial herbivory was incorporated following the tradition in ecological studies aimed to assess the impacts of herbivory on plants (see e.g., Lehtilä & Boalt, 2008; Waterman et al., 2019), due to the experimental advantage of accurately controlling both the amount of physical leaf damage and the chemical effect provided specifically by jasmonic acid (as a surrogate of the more complex insect saliva), that could be uniformly applied to the two

study systems in order to gain generality. In the following sections, I will discuss differences and similarities between results obtained for the two study systems in the patterns of change in DNA methylation recorded and lessons learned from applying different techniques to estimate those methylation changes in DNA in response to herbivory.

DNA METHYLATION LANDSCAPE AND CHANGES AFTER HERBIVORY

Over evolutionary time scales, cytosine DNA methylation has been involved in genome stability and the regulation of gene expression, facilitating phenotypic plasticity in plants and therefore contributing to the potential adaptability of plant populations (as reviewed by Bräutigam & Cronk, 2018). Changes in DNA methylation (also called epimutations) are known to emerge more rapidly than genetic changes, and they can be transmitted to offspring, although some of them are transient (as demonstrated by Becker et al., in 2011 and discussed in a review by Johannes & Schmitz in 2019). Thus, epimutations may serve as a bridge between short-term plastic phenotypic responses and long-term genetic mutations, operating at intermediate time scales that might be relevant to understand microevolutionary process linked to intraspecific variation (Becker et al., 2011; Johannes & Schmitz, 2019; Noshay & Springer, 2021). Also important, DNA methylation is associated with cell and organ differentiation, and other developmental processes related to seasonality and individual aging (see e.g., Alonso et al., 2024 and references therein; Nunez-Martinez et al., 2024). With that background knowledge, we focused on methylation changes observed in DNA of non-damaged leaves of plants which were developed after a first priming event, and fully expanded at collection time (24 h. after second herbivory event), searching for those

epimutations that could be stable enough to be transmitted to offspring (Fitz-James & Cavalli, 2022).

THE COMPARISON BETWEEN INSECT AND ARTIFICIAL HERBIVORY

The two focal species of this Thesis belong to plant families characterized by their ability to produce secondary compounds that are involved in plant defense system against insects and other herbivores, glucosinolates in the Brassicaceae (Mitreiter & Gigolashvili, 2021) and phenolic glycosides in the Salicaceae (Boeckler et al., 2011). These two groups of chemical molecules are highly diverse; thus, the structural complexity and toxicity of different compounds are largely variable (Boeckler et al., 2011; Mitreiter & Gigolashvili, 2021). Plant hormones, particularly the jasmonic acid and its derivatives, have been directly related to the regulation of defense production and individual growth according to environmental conditions and damage recorded (Züst & Agrawal, 2017). In particular, exogenous application of jasmonic acid is able to upregulate the glucosinolate metabolic pathway, altering both composition and concentration of some of those secondary compounds in leaves of some Brassicaceae (Mitreiter & Gigolashvili, 2021; Textor & Gershenzon, 2008). Similar effects have been found in Salicaceae too (Havill & Raffa, 1999).

In this Thesis, we found that artificial damage together with jasmonic acid application can have an impact in leaf DNA methylation in plants with contrasting life-history features and distinct chemical defenses. Furthermore, in *T. arvense*, the effect of insect herbivory was milder in terms of changes in global DNA methylation, glucosinolate concentrations and functional phenotypic traits related to growth and reproductive output (**Chapter 2**),

whereas in *P. nigra* the magnitude of changes in response to insect and artificial herbivory varied geographically (see below and **Chapter 3**). As regards the effects of artificial herbivory, global DNA methylation level in leaves of artificially damaged *T. arvense* plants was reduced compared to methylation level in DNA of undamaged control plants, and an overall upsurge of glucosinolates was observed mainly in late-flowering phenotypes (**Chapter 2**). In the Lombardy poplar, we found that methylation percentage in leaf DNA increased after artificial herbivory mainly in CHH, the sequence context in which many more hyper-methylated cytosines compared to undamaged plants were recorded, frequently associated to transposable elements (TEs) and intergenic regions (**Chapter 3** and **4**). Indeed, we identified 32.7% of differentially methylated cytosines (DMCs) in response to herbivory in TEs, and more than 50% of them overlapped with two TEs families widely abundant in poplar genome, the DNA/Helitron and LTR/Gypsy. These results are congruent with previous studies reporting that non-CG methylation at TEs and repeat intergenic regions are the most dynamic DNA methylation changes in response to stress, and thus, TEs and TE methylation can be a source of hidden variation that conditionally affect the expression of nearby genes (Fitz-James & Cavalli, 2022). Also important, in **Chapter 4**, hypo/hyper DMCs for insect or artificial herbivory were similarly frequent, and treatment-specific DMCs were by far more abundant than non-specific ones (similar output obtained by epiGBS and WGBS). Thus, even though the responses of insect herbivory and artificial herbivory did not provide identical responses *per se*, this PhD Thesis confirms that artificial herbivory can be a valuable tool to gain further mechanistic and epigenetic understanding of plant-herbivore interactions.

Our results pointed to the relevance of distinguishing the sequence context in which methylation varied the most and highlighted that the significance of the effect can vary according to the resolution of the method applied (see below and **Chapter 4**). Indeed, an independent study conducted with *P. nigra* ramets from multiple European provenances that applied WGBS, found that biotic stress, including the exogenous application of salicylic acid and leaf consumption by *Lymantria dispar* caterpillars, reduced methylation in CG and CHG contexts and it had no significant effect in CHH (Peña-Ponton et al., 2024). In that study, drought was the stress with the highest impact in DNA methylation, inducing hyper-methylation at CHH, particularly in TEs located close to drought-responsive genes (Peña-Ponton et al., 2024). Altogether, we can conclude that absence of an overall shift (or little overall variation) in genome-wide DNA methylation levels in response to a certain experimental treatment, may emerge from distinct sign of methylation changes recorded at different sequence contexts and genomic locations (see also Balao et al., 2024). Still, when recorded, the magnitude and sign of change in global DNA methylation can be highly informative because it shows a positive correlation in parent-offspring comparisons (Herrera et al., 2018) and it is frequently related with multiple plant phenotypic traits (see e.g., **Chapter 2**; Alonso et al., 2014, 2018; Herrera & Bazaga, 2013).

THE EFFECT OF ARTIFICIALLY ALTERING DNA METHYLATION WITH 5-AZACYTIDINE IN THLASPI ARVENSE

Experimental alteration of DNA methylation by using 5-Azacytidine (5-AzaC), an inhibitor of the activity of a DNA methyltransferase enzyme, has contributed to uncover the effect of this element of epigenetic regulation on phenotype, development and fitness in different species (see e.g., Herman &

Sultan, 2016; Rendina González et al., 2016; Puy et al., 2018; Münzbergová et al., 2019; Herrera et al., 2021; Alonso et al., 2024). As a general rule, 5-AzaC applied at high concentration during seed germination may be toxic and seriously impair individual growth (Puy et al., 2018). However, when used at low concentration and during short time, 5-AzaC slows individual growth, delays leaf production and flowering, and may incur some negative impact on fitness (Alonso et al., 2017, 2024 and references therein; Bossdorf et al., 2010). It can also modify the patterns of phenotypic plasticity helping to understand the links between genetics, epigenetics and functional phenotype (Bossdorf et al., 2010; Herman & Sultan, 2016; Verhoeven & van Gurp, 2012). In *T. arvense*, the effect 5-AzaC was still evident as a reduction of DNA methylation in leaves of reproductive individuals but only in the early-flowering ecotype (**Chapter 2**), suggesting that the genetic background of experimental plants can affect the response to this enzymatic inhibitor and the subsequent phenotypic changes (delayed growth and reduced size). Thus, a significant and homogeneous effect of seed demethylation treatments cannot be presumed across individuals, populations or species. A better molecular understanding of the effects 5-AzaC in different plant species and tissues could help to understand the observed heterogeneity (see Balao et al., 2024; Griffin et al., 2016 and references therein).

THE EPIGENETIC RESPONSE CAN VARY WITH PLANT PROVENANCE

As mentioned above, the response to experimental herbivory was not uniform in any of the two study systems. In *T. arvense* (**Chapter 2**), the two flowering ecotypes (Mulligan & Kevan, 1973) collected from distant European populations in Germany (early-flowering) and Sweden (late-flowering), which belonged to different genetic clusters according to Galanti

et al. (2022), were not equally affected by herbivory and 5-AzaC. In the group of non-treated seeds, early-flowering plants were the least affected by herbivory, whereas late-flowering plants reduced DNA methylation level in leaves, increased leaf glucosinolate concentration (particularly sinigrin) and reached reduced final stem size after damage. In contrast, early-flowering plants were the most responsive to 5-AzaC, significantly reducing DNA methylation and growth. Differential epigenetic response after experimental stress treatments of plant genotypes, lines or provenances have been previously reported (see e.g., Bossdorf et al., 2010; Herman & Sultan, 2016; Münzbergová et al., 2019); although a deeper understanding of the mechanisms behind is still missed. For instance, the observed variation in epigenetic response and phenotypic consequence could be attributed to either the parental generation's experience (Latzel et al., 2023), together with difference in functional traits and flowering time among populations, which are integral to local adaptation mechanisms and may be influenced by variations in the climate of the native habitat (Bussotti et al., 2002; Ramírez-Valiente et al., 2010; Kaluthota et al., 2015; Ramírez-Valiente & Cavender-Bares, 2017).

The identification of origin-specific, intraspecific epigenetic modifications triggered by herbivory in the clonal *P. nigra* was an unexpected finding (**Chapter 3**). We found that the methylation changes in response to herbivory of near-isogenic poplars from three European populations (Spain, Italy, Poland) were not homogeneous. Random epigenetic mutations could be a relevant source of epigenetic variation in this clonally propagated tree as suggested by a parallel common garden study conducted with multiple European provenances of Lombardy poplar (Díez Rodríguez et al., 2022b) and could be likely related to the epigenetic differentiation observed in

control undamaged plants (**Chapter 3**). Furthermore, studies conducted to explore natural epigenetic variation along climatic gradients in this and other tree species, suggest that population epigenetic differentiation may be correlated to some extent with climatic conditions of the study plants or their provenances (Gugger et al., 2016; Díez Rodríguez et al. 2022a, Galanti, et al., 2022; Guevara et al., 2022), and highlighted the significance of epigenetic variation in natural plant populations with contrasting environmental conditions (Gao et al., 2010; Herrera & Bazaga, 2011; Raj et al., 2011; Richards et al., 2012; Guevara et al., 2022). This source of epigenetic variation could be also relevant in our study because we selected source populations with contrasting climates and methylation in CHH varied geographically in *P. nigra*, mainly associated to extreme temperatures (e.g., Tmax warmest month, Tmin coldest month) and seasonality (Díez Rodríguez et al. 2022a; see also Galanti, et al., 2022 for pennycress). Altogether, the results presented in this Thesis indicated that notable intraspecific divergence in the response to herbivory were detected in two plant species differing in life history, genetic variance and chemical defense system. Further studies (e.g., epiQTL) are needed to gain insight on the underlying molecular mechanisms which could lead to identify potential (epi)genomic targets for enhancing herbivore tolerance in crop plants or forest management (Springer & Schmitz, 2017).

PROGRESS AND CHALLENGES IN REVEALING DNA METHYLATION VARIATION IN COMPLEX PLANT GENOMES

Plant genomes are outstandingly variable in size and complexity. Such diversity is largely related to the abundance of TEs and the frequency of whole-genome duplication and hybridization events that largely characterize the evolution of the youngest and currently most diverse group of land plants,

the Angiosperms (Springer et al., 2016; Soltis & Soltis, 2021). As mentioned above, several interrelated epigenetic mechanisms play regulatory roles in providing stability to plant genomes, including DNA methylation, histone modifications and small RNAs (Matzke & Mosher, 2014; Pikaard & Mittelsten Scheid, 2014; Saze et al., 2012). This Thesis focused on DNA methylation, a key element that is largely variable among plant species, with a strong phylogenetic signal and correlated evolution with haploid genome size (Alonso et al., 2015, 2019; Bewick & Schmitz, 2017), and has been very frequently studied in Ecological Epigenetics (Richards et al., 2017).

For the experimental part, I have used three different methods for estimating changes in DNA methylation: a global estimation based on chromatography (**Chapter 2**); and two high-resolution sequencing techniques after bisulfite conversion of extracted DNA (**Chapters 3** and **4**), which provide single nucleotide methylome information in reduced genomes (epiGBS) or entire genomes (WGBS). Methods were selected according to my questions and available resources, with resources meaning (i) time and budget to process samples, (ii) training and time required for obtaining sound results and (iii) public availability of necessary bioinformatic tools and genomic resources (see **Box 1** at General Introduction for details). Getting high-resolution data for ecological studies was challenging seven years ago when this project was conceived, particularly if working with plants lacking a fully annotated reference genome (that was the case of *T. arvense* when we started) or in experimental settings that required a large number of replicates (Richards et al., 2017). Indeed, in some cases it remains challenging, as we did not succeed in producing good-quality libraries for epiGBS analysis of *Fragaria vesca* grown from seeds under controlled conditions with a factorial design that, similar to the one applied to *T. arvense*, included two treatments:

seed demethylation with 5-AzaC (control and demethylated) and herbivory (natural herbivory, artificial herbivory and control plants). Such failure could be likely due to the high polysaccharide and polyphenol content in *F. vesca* leaves, which increases sample viscosity and reduces DNA quality, ultimately interfering with PCR performance (Nunes et al., 2011).

Earlier studies in Ecological Epigenetics were mainly based on the analysis of methylation sensitive amplified fragment length polymorphisms (MS-AFLP or MSAPs) that are anonymous markers (Richards et al., 2012; Alonso et al., 2016) and offered the possibility to generate numerous polymorphic bands per reaction without prior knowledge of the genomic sequence. This technique can be applied to a large number of individuals and populations, producing individual fingerprints and accurate estimates of epigenetic variation within plant populations, suitable to investigating patterns of spatial differentiation of non-model organisms growing in the wild (see e.g., Herrera & Bazaga, 2010 for *Viola cazorlensis*; Medrano et al., 2020 for a comparison involving many endemic and non-endemic plant species). These methods contributed to uncover that some plant species with minimal genetic variation exhibited a high degree of methylation variation, which could enhance fitness (e.g., in *Pinus pinea*, Sáez-Laguna et al., 2014) and be linked to habitat differentiation (e.g., in *Spartina alterniflora*, Foust et al., 2016). Furthermore, MSAPs were able to distinguish between changes occurring in CG and CHG sequence contexts and were frequently used to exploring changes induced by abiotic stress (reviewed in Alonso et al., 2016). Although MSAPs have limitations to quantify the magnitude of methylation change and do not provide information as regards the genomic features involved (Alonso et al., 2016), they are still in use and can be particularly suitable to investigate the relationships between genetic and epigenetic variation in natural plant

populations (Herrera et al., 2016; Valverde et al., 2024). Similar to MSAPs, global DNA methylation estimates obtained by HPLC do not provide information of the context or genomic location where methylation occurred. However, these estimates can be very useful in comparative approaches (see e.g., Alonso et al., 2019) and to offer insight into intraspecific variation in the effect of experimental demethylation that can be otherwise overlooked (see **Chapter 2**, and Alonso et al., 2024). The technique requires some expensive equipment and technical expertise to run it that was available in my host institution making it affordable for my studies.

In **Chapter 4**, I addressed a long-standing issue for ecologists, examining suitability of emerging bioinformatics tools to study DNA methylation changes in response to a certain stress factor at single-nucleotide resolution, even in species for which no reference genome is available (Schield et al., 2016; Trucchi et al., 2016; van Gurp et al., 2016; Gawehns et al., 2022), in comparison to the gold standard and more expensive option of WGBS, which strictly required a reference genome (Suzuki et al., 2018). We experimentally evaluated the outcome and biological interpretation of epiGBS (with and without using reference genome) and WGBS techniques, in experimental herbivory experienced by a plant with limited genetic variation, the 'italica' clone of *P. nigra*. We concluded that epiGBS offered reliable insight into methylation changes in the DNA of poplar clones experiencing insect and artificial herbivory. The two techniques showed increased methylation mainly in CHH and agreed on the specificity of the response elicited by insect and artificial herbivory. DMCs captured by the two techniques were mainly associated to TEs and intergenic regions for CHH, and to gene bodies and their flanking regions in CG and CHG. Thus, epiGBS succeeded to characterize genome-wide methylation changes in response to

herbivory and could be, thus, useful for investigating responses to stress factors in species lacking a reference genome. Still, geographic variation in the output obtained by epiGBS, suggested this method might bear some limitations in providing functional interpretation of recorded methylation changes (at the level of Gene Ontology terms), a drawback to characterize the epigenetic regulation of stress-specific responses in non-model plants, that could be relieved by simultaneous transcriptome analyses.

IMPLICATIONS FOR FUTURE RESEARCH

This PhD Thesis focused predominantly and purposefully on DNA cytosine methylation due to its well-known immediate and long-term effects in response to stress (Bossdorf et al., 2010; Herman & Sultan, 2016). However, it does not rule out that other epigenetic mechanisms along with genetic variation may play a role in shaping plant-herbivore interactions (Colicchio et al., 2018; Colicchio & Herman, 2020). Thus, analyzing the response of other epigenetic processes such as histone modification, chromatin configuration and small RNAs, as reported in **Chapter 1**, could offer new insight in understanding induced plant defenses and plant-herbivore interactions (Matzke & Mosher, 2014; Saze et al., 2012). Although these mechanisms were not studied in this dissertation, they may provide valuable insights for future research.

Furthermore, we have not assessed the adaptive significance of transgenerational transmission of the epigenetic response to herbivory in plants due to time constraints. Changes in DNA methylation, as indicated by several studies on different stress factors (Johannes et al., 2009; Verhoeven et al., 2010; Van Antro et al., 2023), can be passed across plant generations,

suggesting their potential role in plant adaptation to different biotic and abiotic stresses and long-term evolution (Burggren, 2016). Analyzing not only epigenetic variation but also transgenerational inheritance behind variation in specific plant defense traits can help us understand and predict evolution by connecting methylation variation and phenotypic variation (Colicchio et al., 2015, 2018; Colicchio & Herman, 2020). Along with incorporating epigenetics into evolutionary models (Geoghegan & Spencer, 2012; Kronholm & Collins, 2016), more empirical data from natural plant populations is required for accurate modelling the epigenetic dynamics associated to plant-herbivory interactions.

In summary, the compendium of the studies presented in my PhD Thesis suggest that future research on epigenetic contribution to plant-herbivore interactions should take five key factors into account. (i) Assessing multiple species, including a range of families, genotypes and populations experiencing contrasting regimes of herbivory in nature. (ii) Analyzing progenies of plants experiencing contrasting herbivory damage derived both as seeds or clones to understand the intergenerational effects. (iii) Designing studies integrating several "omic" techniques (e.g., methylome, transcriptome, proteome) to gain a comprehensive understanding of the molecular mechanisms that regulate plant responses to herbivory. (iv) Creating new epigenetic resources such as epialleles, epigenetic recombinant inbred lines (epiRILs), epigenetic quantitative trait loci (epiQTLs), and epigenetic hybrids (epihybrids) in other species than *Arabidopsis* to understand stress tolerance which could be beneficial in crop breeding or forest management. (v) Evaluating the relevance of epigenetic variation within individual plants in response to herbivory.

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GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

This thesis combined a literature review with novel experimental contributions to investigate the epigenetic contribution to plant defense in response to insect herbivory and reached the following conclusions:

1. Previous publications highlighted the role of epigenetics in regulating plant-biotic interactions but there is limited evidence regarding insect herbivory compared to the more frequently studied interactions with bacteria and fungi.
2. Changes in DNA methylation induced by insect and artificial herbivory were not identical. Artificial herbivory treatments including exogenous application of jasmonic acid, can have an impact in leaf DNA methylation in plants with contrasting chemical defenses and life-history features.
3. Global DNA methylation in leaves of the annual *Thlaspi arvense* decreased after experimental herbivory. The magnitude of change was bigger for artificial herbivory and varied with seed provenance associated to genetic background and life-cycle phenology.
4. The two ecotypes of *Thlaspi arvense* showed divergent patterns of phenotypic plasticity to cope with herbivory. Late-flowering plants increased aliphatic glucosinolates concentration and reduced individual biomass, whereas early-flowering plants were less affected except for a reduction in seed mass.
5. The effects of demethylation with 5-azacytidine applied during seed germination can vary with seed provenance and, thus, it cannot be presumed uniform across studies.

6. In Lombardy poplar trees, herbivory induced context- and herbivory-type specific methylation changes in leaf DNA. Cytosine methylation was significantly increased in the CHH context, with Differentially Methylated Cytosines in this context being predominantly located at intergenic regions.
7. Almost one third of Differentially Methylated Cytosines induced by herbivory in poplars overlapped with transposable elements, particularly with the gypsy retrotransposons (LTR-Gypsy) and the DNA-Helitron families.
8. Differential methylation in response to experimental herbivory was heterogeneous among poplar tree provenances, suggesting that random epigenetic mutations could be a relevant source of epigenetic variation in clonally propagated trees.

9. Genome-wide methylation alterations in response to herbivory can be appropriately characterized by different methods. Distinguishing the sequence context and genomic location of the changes recorded helps to better interpret the results.

10. Reduced Representation Bisulfite Sequencing (RRBS) techniques should be particularly useful for large-scale ecological epigenetic studies of species lacking a reference genome. Functional interpretation might require complementary transcriptome analyses

CONCLUSIONES GENERALES

Esta Tesis Doctoral combinó una revisión de la literatura científica con el desarrollo de una parte experimental original para investigar la contribución epigenética a la defensa de las plantas en respuesta a la herbivoría por insectos y llegó a las siguientes conclusiones:

1. Publicaciones anteriores destacaron el papel de la epigenética en la regulación de las interacciones bióticas entre plantas y otros organismos. Sin embargo, la evidencia es más limitada para las interacciones de herbivoría por insectos, en comparación con las interacciones con bacterias y hongos.
2. Los cambios en la metilación del ADN inducidos por la herbivoría realizada por insectos y la herbivoría artificial no fueron idénticos. Los tratamientos de herbivoría artificial que incluyen la aplicación exógena de ácido jasmónico logran tener un impacto en la metilación del ADN de las hojas, en especies con historias de vida y defensas químicas diferentes.
3. La metilación global en el ADN de las hojas de *Thlaspi arvense* disminuyó después de la herbivoría experimental. La magnitud del cambio fue mayor para la herbivoría artificial, y varió con la procedencia de las semillas, en ecotipos con divergencia en factores genéticos y fenología del ciclo de vida.
4. Los dos ecotipos de *Thlaspi arvense* mostraron patrones divergentes de plasticidad fenotípica para hacer frente a la herbivoría. Las plantas de

floración tardía aumentaron la concentración de glucosinolatos alifáticos y redujeron la biomasa individual, mientras que las plantas de floración temprana se vieron menos afectadas, excepto por una reducción en el tamaño de sus semillas.

5. Los efectos de la desmetilación con 5-azacitidina aplicada en la etapa de germinación de la semilla pueden variar según la procedencia de la semilla y, por lo tanto, no se puede asumir que sean uniformes en todos los estudios.
6. En *Populus nigra* 'italica', la herbivoría indujo cambios de metilación en el ADN de las hojas que son específicos del tipo de herbivoría y de diferente magnitud según el contexto de secuencia analizado. La metilación aumentó especialmente en el contexto CHH, y predominantemente en regiones intergénicas.
7. En esta misma especie, casi un tercio de las citosinas con cambios significativos de metilación inducidos por herbivoría se superpusieron con elementos transponibles, particularmente con dos familias muy abundantes en el genoma, los retrotransposones LTR-Gypsy y los DNA-Helitron.
8. La metilación diferencial en respuesta a la herbivoría experimental fue heterogénea entre las procedencias de los álamos lombardos, lo que sugiere que las epimutaciones aleatorias podrían ser una fuente relevante de variación epigenética en árboles propagados clonalmente.
9. Las alteraciones de la metilación en respuesta a la herbivoría que suceden a lo largo del genoma pueden caracterizarse adecuadamente mediante diferentes métodos. Distinguir el contexto de secuencia y la

ubicación genómica de los cambios registrados ayuda a interpretar mejor los resultados.

10. Las técnicas de representación reducida basadas en secuenciación con bisulfito (en inglés RRBS) se encontraron particularmente útiles para analizar cambios de metilación en estudios ecológicos a gran escala y cuando implican especies que carecen de un genoma de referencia. La interpretación funcional podría requerir análisis de transcriptoma complementarios.

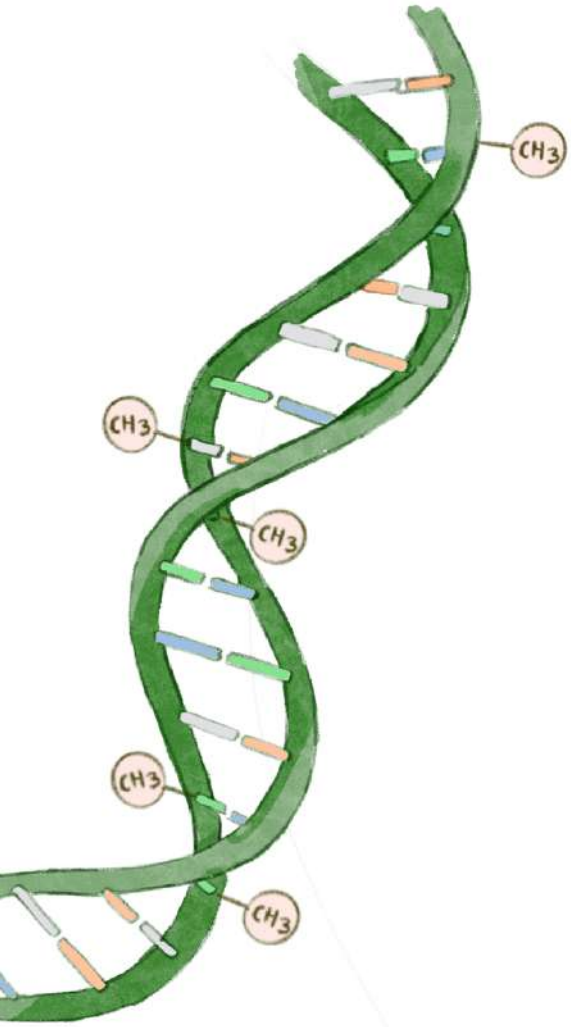
CONTRIBUTION TO SCIENCE COMMUNICATION

I did my PhD on the role of epigenetics in plant-herbivore interactions. There are limited resources for this topic because it is at the heart of a very new area of ecological plant epigenetics. PhD science outreach is crucial because science outreach improves public scientific knowledge. It not only improves communication skills, helps but also helps early stage researchers to communicate knowledge, and instills a feeling of public participation, which scientists increasingly value. Therefore, it is more important than ever that I disseminate the skills and knowledge I learned throughout my PhD to help this subject develop. Because of this, I have participated in a number of scientific outreach activities, such as contributing a chapter to a textbook on ecological plant epigenetics and doing public outreach about my PhD research (https://www.youtube.com/watch?v=BYXRxdrXwRs).

I co-authored this textbook with other members of the EpiDiverse Training Network in order to disseminate the information and resources that we lacked when we first began our work. As a new growing fields, comprehensive theoretical knowledge and bioinformatics resources are obtainable for scientists. This textbook covers a wide range of topics from a variety of angles, including ecological, molecular, and bioinformatics. In order to have a complete picture of the plant defense response, I wrote the second chapter of the Ecology part, titled "Plant Defense Response," for which I am mostly responsible (accessible at: https://epidiverse.gitbook.io/project/-MfxkdBDZggX_vc_sG5l/, accessed on 20 May 2023).As part of the MSCA ITN project EpiDiverse, I also received training and experience in a professional scientific communication setting at a company in Berlin, Germany called Wissenschaft im Dialog gGmbH. Markus Weißkopf and Jona Adler served as the supervisors.

As a part of the European Project EpiDiverse and Estación Biológica de Doana-CSIC, I helped to organize and develop the content of an online Kahoot quiz for

kids entitled "EpiDiver: Play with epigenetics and learn about plants" for the European Researchers Night on November 27, 2020 (virtually) where 500 People took part. Furthermore, I gave a talk at the CEIP Pedro Garfias in Sevilla, Spain, on 11-February-2020 (4 hours) in honor of "International day for women and girls in science - 11th February." The Estación Biológica de Doana-CSIC hosted a children's workshop titled "Bryophytes: those little strangers" as part of the European Researchers Night on September 28 (for an hour) in Sevilla. 2019: Take part in an outreach video interview to be posted on the YouTube channel for CSIC Andaluca y Extremadura (https://www.youtube.com/watch?v=_Ns_BrvvjnQ) and participated on the "Yo investigo and was a finalist for the "Yo soy CSIC" 2019 Award given by the DPE of the CSIC (Consejo Superior de Investigaciones Cientficas) (https://youtu.be/IACgkyiZyfk)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