

**DNA methylation dynamics in *Brassica napus* under combined drought and *Verticillium*
longisporum stress**

by

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ABSTRACT

Brassica napus L. (canola) is a major oilseed crop whose productivity is increasingly threatened by vascular pathogens such as *Verticillium longisporum* (Stark) Karapapa, Bainbridge & Heale. While plant responses to pathogen infection have been widely studied, the role of epigenetic regulation, particularly DNA methylation, in modulating these responses remains poorly understood. Furthermore, how environmental stresses such as drought influence epigenetic and molecular responses during infection is not well characterized. This thesis aimed to investigate the role of DNA methylation in *B. napus* under *V. longisporum* infection and to examine how combined drought and pathogen stress influence molecular defence mechanisms. Using Oxford Nanopore Technologies long-read sequencing, genome-wide cytosine methylation profiling was performed to identify epigenetic changes associated with pathogen infection. Differential methylation analysis revealed that methylation changes were predominantly localized and occurred mainly in CG and CHG contexts, with comparatively limited changes in the CHH context. Some of the differentially methylated loci were associated with genes involved in plant defence, including those related to pathogen perception, signalling, and stress responses, suggesting a role for DNA methylation in regulating targeted defence mechanisms. To further investigate stress interactions, molecular responses of *B. napus* hypocotyl tissues to *V. longisporum* infection under contrasting water regimes were examined. Drought influenced structural defence mechanisms, including enhanced lignin and suberin deposition and modulation of cell wall-associated gene expression, contributing to reduced pathogen progression. Complementary DNA methylation profiling at the early stage of stress exposure suggested that epigenetic modifications may contribute to the establishment of these transcriptional responses under combined stress conditions. Overall, DNA methylation in *B. napus* is dynamically and context-specifically regulated during *V. longisporum* infection and drought stress, supporting potential roles in defence responses and stress resilience in polyploid crops.

FOREWORD

This thesis follows the manuscript format specified by the Department of Plant Science and the Faculty of Graduate Studies at the University of Manitoba. The citations and reference style in this document followed the American Psychological Association 7th edition. This thesis includes an abstract, an introduction, a literature review, two manuscripts, each with an abstract, introduction, materials and methods, results, discussion, and conclusion sections, and a conclusion and future research recommendations section. Supplementary data is provided after the main body of the thesis.

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1.INTRODUCTION

Brassica napus L. is one of the most important oilseed crops worldwide and serves as a major source of edible oil, livestock feed, and industrial raw materials (Friedt & Snowdon, 2010; Mohamed et al., 2025). In Canada, canola contributed approximately 43.7 billion CAD to the national economy in 2023, underscoring its economic importance (Canola Council of Canada, 2024). However, its production is increasingly challenged by both abiotic and biotic stresses, particularly under changing climatic conditions that expose crops to multiple environmental constraints simultaneously (IPCC, 2021; Ramegowda & Senthil-Kumar, 2015).

Among abiotic stresses, drought is considered one of the most significant factors limiting crop productivity worldwide. Water deficit disrupts plant water relations, photosynthesis, nutrient transport, and cellular metabolism, ultimately reducing growth and yield (Elferjani & Soolanayakanahally, 2018; Lawlor & Cornic, 2002). *Brassica napus* is particularly sensitive to water limitation. In 2021, drought conditions in Western Canada reduced national *B. napus* production by 35.4% to 12.6 million tonnes, the lowest national yield recorded since 2007 (Statistics Canada, 2021). At the physiological level, drought stress can severely impair photosynthetic performance and reproductive development, leading to substantial reductions in seed production and oil yield (Bouchyoua et al., 2026; Elferjani & Soolanayakanahally, 2018). Concurrently, *B. napus* production is threatened by soil-borne pathogens, among which *Verticillium longisporum* (Stark) Karapapa, Bainbridge & Heale, the causal agent of Verticillium stem striping, has emerged as an important vascular pathogen of *Brassica* crops (Depotter et al., 2016; Heale & Karapapa, 1999). Following root infection, the pathogen colonizes xylem tissues, spreads systemically throughout the host, and can cause yield losses of up to 50% in heavily infected crops (Cui et al., 2025; Dunker et al., 2008; Eynck et al., 2007).

Although drought and pathogen infection are often studied independently, crops in agricultural systems are rarely exposed to a single stress factor in isolation. Combined abiotic and biotic stresses elicit physiological, molecular, and transcriptional responses that differ substantially from those induced by individual stresses alone (Atkinson & Urwin, 2012; Ramegowda & Senthil-Kumar, 2015; Suzuki et al., 2014). Consequently, understanding how drought influences plant-pathogen interactions has become an important research priority. Previous studies have shown that drought can either enhance resistance or increase susceptibility depending on the host-pathogen system, environmental conditions, and timing of stress exposure (Atkinson & Urwin, 2012; Rivero

et al., 2022). In the *B. napus*-*V. longisporum* pathosystem, recent work from our laboratory highlighted that drought stress (30% field capacity) reduced the acropetal movement of the pathogen from roots to hypocotyl tissues and decreased disease severity, indicating that water availability strongly influences the progression of vascular colonization (Kashyap et al., 2026). However, the molecular mechanisms underlying this drought-mediated suppression of disease remain poorly understood.

Plant responses to environmental stress are coordinated through complex regulatory networks involving hormonal signalling, transcriptional reprogramming, metabolic adjustments, and defence activation (Pandey et al., 2015; Suzuki et al., 2014). Epigenetic regulation has emerged as an important component of these adaptive responses. Epigenetic mechanisms modify gene activity without altering the underlying DNA sequence and include DNA methylation, histone modifications, chromatin remodelling, and non-coding RNAs (Bird, 2007; Law & Jacobsen, 2010; Zhang et al., 2018). Among these mechanisms, DNA methylation is particularly important because of its roles in regulating gene expression, transposable element activity, and genome stability. Studies in *Arabidopsis thaliana* have showed that disruption of DNA methylation results in widespread activation of normally silenced transposable elements, highlighting its importance in maintaining genome integrity (Bewick & Schmitz, 2017; Liang et al., 2022).

Numerous studies have evidenced that DNA methylation is responsive to both abiotic and biotic stresses. In *Oryza sativa* L. (rice), drought stress induces genome-wide methylation and demethylation events, some of which persist after stress recovery, suggesting that DNA methylation may contribute to longer-term stress memory (Wang et al., 2011). Such findings have motivated a growing interest in DNA methylation as a mechanism linking stress perception to adaptive gene regulation and stress memory more broadly (Crisp et al., 2016; Lämke & Bäurle, 2017). Despite this evidence, most studies have focused on individual stress factors, and comparatively little is known about methylation dynamics during combined abiotic and biotic stresses, particularly in polyploid crop species such as *B. napus* (Chalhoub et al., 2014; Zhang et al., 2018).

Advances in long-read sequencing technologies have substantially improved the ability to investigate epigenetic variation in complex plant genomes. Oxford Nanopore Technologies sequencing enables direct detection of DNA methylation from native DNA molecules while simultaneously generating nucleotide sequence information, eliminating the need for chemical

conversion or amplification-based approaches (Gouil & Keniry, 2019; Sigurpalsdottir et al., 2024). These capabilities are particularly advantageous for polyploid genomes such as *B. napus*, which possess extensive repetitive regions and duplicated genomic sequences that can complicate conventional methylation analyses (Amarasinghe et al., 2020; Chalhoub et al., 2014).

The present study investigates genome-wide DNA methylation dynamics associated with *V. longisporum* infection in *B. napus* and examines how these responses are altered under combined drought and pathogen stress conditions. By integrating Oxford Nanopore Technologies-based methylome profiling with complementary transcriptomic analyses, this research aims to identify epigenetic and transcriptional changes associated with pathogen colonization, drought-mediated suppression of disease development, and plant adaptation to combined stress conditions. Ultimately, this work contributes to a broader understanding of stress regulation in polyploid crop species and provides new insights into the role of epigenetic mechanisms in coordinating plant responses to complex environmental challenges.

2. LITERATURE REVIEW

2.1 *Brassica napus*: Origin, Agricultural Importance, and Production Constraints

The Brassicaceae family comprises approximately 340 genera and 4,636 species distributed across 52 tribes (Francis et al., 2021). Several members of the family have been domesticated for vegetable, forage, condiment, and oilseed production, including species within the genus *Brassica* (Warwick, 2011). Among these, *Brassica napus* L. is cultivated primarily as an oilseed crop, providing edible oil for human consumption and protein-rich meal for livestock feed, while also serving as a feedstock for biofuel and industrial applications (Mohamed et al., 2025). The agronomic significance of *B. napus* is closely tied to its evolutionary origins, which involved natural interspecific hybridization events that gave rise to the modern amphidiploid *Brassica* crops cultivated today (Chalhoub et al., 2014). The genetic relationships among cultivated *Brassica* species were first formalized by Nagaharu U through the Triangle of U model, which explains how three diploid progenitor species, *Brassica rapa* L. (AA), *Brassica nigra* (L.) W.D.J. Koch (BB), and *Brassica oleracea* L. (CC), gave rise to three allotetraploid species through natural hybridization and chromosome doubling (Nagaharu U, 1935). Among these allotetraploids, *B. napus* possesses the AACC genome and originated through hybridization between *B. rapa* and *B. oleracea* (Chalhoub et al., 2014). The origin of *B. napus* is estimated to have occurred within the last 7,500 years, and subsequent genome duplication, chromosomal rearrangements, and gene retention events have resulted in a complex polyploid genome containing numerous duplicated genes (Chalhoub et al., 2014; Song et al., 2020).

Early rapeseed cultivars contained approximately 45-55% erucic acid as a proportion of total seed oil fatty acids, as well as high concentrations of glucosinolates in the seed meal, traits that limited their suitability for human consumption and livestock feeding (Bell, 1982). Through extensive breeding efforts, cultivars containing less than 2% erucic acid in the oil and less than 30 $\mu\text{mol g}^{-1}$ glucosinolates in the meal were developed, resulting in the canola-quality varieties cultivated today (Canola Council of Canada, 2025a; Stefansson & Kondra, 1975). These improvements substantially enhanced the nutritional quality and commercial value of *B. napus*, driving its widespread adoption as one of the most important oilseed crops in global agriculture (Friedt & Snowden, 2010).

The economic importance of *B. napus* is particularly evident in Canada, where the crop plays a central role in agricultural production and international trade. Canada is the world's largest exporter of canola, with production concentrated primarily in the Prairie provinces of Saskatchewan, Alberta, and Manitoba, which account for approximately 99% of national output (Canola Council of Canada, 2025b). In 2023, *B. napus* generated approximately 43.7 billion CAD in economic activity and supported more than 206,000 jobs across Canada, underscoring its importance to the national economy (Canola Council of Canada, 2024). Despite these achievements, maintaining stable *B. napus* production is increasingly challenged by environmental stresses, among which drought is considered one of the most significant constraints to agricultural productivity worldwide (Seleiman et al., 2021). *B. napus* is particularly sensitive to water limitation, with drought stress reducing net photosynthetic assimilation rate and stomatal conductance significantly (Ahmar et al., 2019). In controlled studies, stomatal conductance declined from $0.52 \text{ mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ under well-watered conditions to $0.14 \text{ mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ under drought, driven primarily by abscisic acid ABA-induced stomatal closure that limits CO_2 diffusion and carbon assimilation (Elferjani & Soolanayakanahally, 2018). These consequences are especially pronounced during reproductive development, where water deficit can reduce pod formation and seed yield (Elferjani & Soolanayakanahally, 2018).

In addition to abiotic stresses, diseases caused by soil-borne fungal pathogens represent a critical and persistent constraint to *B. napus* productivity. Among the soil-borne pathogens affecting *B. napus*, *Verticillium longisporum* (Stark) Karapapa, Bainbridge & Heale, the causal agent of Verticillium stem striping, is considered one of the most economically important vascular pathogens of this crop (Depotter et al., 2016; Heale & Karapapa, 1999). The pathogen survives in soil as melanized microsclerotia and colonizes the vascular tissues of its host following root infection, where it disrupts water transport and impairs plant growth (Depotter et al., 2016; Eynck et al., 2007; Sarenqimuge et al., 2025). Because of its persistence in agricultural soils, widespread distribution, and potential to cause substantial yield losses of up to 50% in heavily infected crops, *V. longisporum* has become an increasing concern in major *B. napus* growing regions (Depotter et al., 2016; Dunker et al., 2008).

2.2 *Verticillium longisporum* and Verticillium Stem Striping Disease

Species belonging to the genus *Verticillium* are important vascular pathogens that infect numerous crop species and cause substantial economic losses worldwide (Fradin & Thomma, 2006; Klosterman et al., 2011). Members of this genus colonize the vascular tissues of their hosts, disrupting water transport and ultimately reducing plant growth, vigor, and productivity (Schnathorst, 1981; Tian & Kong, 2022). While *Verticillium dahliae* Kleb. possesses a broad host range encompassing more than 400 plant species (Tian & Kong, 2022), *V. longisporum* is primarily associated with members of the Brassicaceae and is regarded as one of the most important vascular pathogens affecting *B. napus* production (Depotter et al., 2016; Inderbitzin et al., 2011).

The evolutionary origin of *V. longisporum* distinguishes it from most other species within the genus. Morphological and molecular analyses established *V. longisporum* as a distinct species pathogenic to *B. napus* (Karapapa et al., 1997). Subsequent genomic studies demonstrated that the pathogen originated through three independent interspecific hybridization events involving distinct ancestral lineages, giving rise to the A1/D1, A1/D2, and A1/D3 lineages that differ in their genomic composition and host associations (Inderbitzin et al., 2011). The hybrid origin is thought to have contributed to the adaptation of *V. longisporum* to Brassicaceae hosts and distinguishes it from other *Verticillium* species that infect a broader range of plant families (Depotter et al., 2016; Inderbitzin et al., 2011). Consequently, *V. longisporum* became an important pathogen of *B. napus* in Europe and has more recently been reported in North American production systems (Heale & Karapapa, 1999; Wang et al., 2024).

In *B. napus*, *V. longisporum* infection is characterized by a prolonged asymptomatic phase before the appearance of stem striping symptoms later in plant development (Eynck et al., 2007). This latent infection phase presents a significant challenge for disease management because infected plants may appear healthy while fungal colonization continues within vascular tissues (Depotter et al., 2016; Eynck et al., 2007). The disease cycle begins with the germination of microsclerotia, melanized survival structures that persist in soil following the decomposition of infected crop residues (Sarenqimuge et al., 2024). Long-term field experiments have exhibited that a viable fraction of microsclerotia can persist in soil for around 8 years, allowing the pathogen to survive between growing seasons and making eradication from infested fields particularly difficult (Sarenqimuge et al., 2025). Root exudates released into the rhizosphere promote germination of *Verticillium* microsclerotia by alleviating bacterial suppression in the soil environment, thereby

facilitating the initiation of root infection (Sarenqimuge et al., 2024). Following penetration, the pathogen advances through the root cortex and reaches the xylem vessels, where systemic colonization of the host is initiated (Eynck et al., 2007). Once established within the vascular system, *V. longisporum* produces conidia that are transported upward through the transpiration stream, enabling the fungus to colonize aboveground tissues (Johansson, 2006). During the later stages of infection, extensive microsclerotia formation occurs within senescing stem tissues (Eynck et al., 2007; Eynck et al., 2009). Following crop residue decomposition, these structures are released back into the soil, thereby completing the disease cycle and serving as a source of inoculum for subsequent growing seasons (Sarenqimuge et al., 2025). The key stages of infection, colonization, and survival that comprise the *V. longisporum* disease cycle are illustrated in Figure 2.1.

Verticillium Stripe Disease Cycle

(Caused by the fungus *Verticillium longisporum*)



4 Diseased Canola Plant

The fungus (*Verticillium longisporum*) inhibits regular flow of nutrients and water, causing the xylem to eventually turn black and collapse.

Canola plants display symptoms of leaf chlorosis, early ripening, stunting, necrosis, and shredding or striping of stem tissue. See photos below.

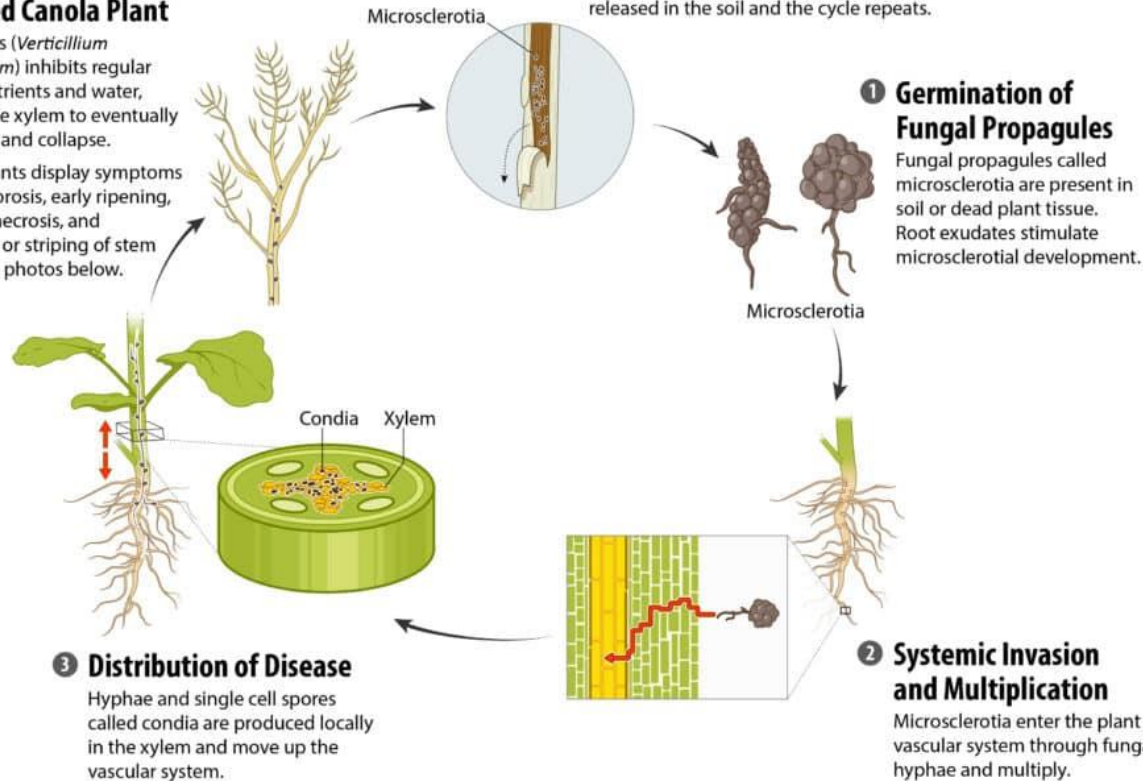


Figure 2.1 Disease cycle of *Verticillium longisporum* in canola, illustrating microsclerotia survival in soil, root infection, systemic colonization through the xylem, disease development, and microsclerotia formation and return to soil (used with permission from Canola Council of Canada).

In *Brassica* crops, the most characteristic symptom of *V. longisporum* infection is the appearance of dark longitudinal stripes on mature stems, a condition commonly referred to as Verticillium stem striping (Depotter et al., 2016; Heale & Karapapa, 1999). Infected plants may also exhibit premature senescence, stunting, reduced vigor, and yield losses with disease severity generally increasing as systemic fungal colonization progresses (Dunker et al., 2008; Eynck et al., 2009). Histological examinations have revealed the presence of microsclerotia within stem tissues during advanced stages of infection. Microsclerotia are compact, melanized aggregates of hyphal cells that form within host vascular and cortical tissues as the fungus transitions from active colonization to a reproductive and survival phase (Heale & Karapapa, 1999). Their characteristic black pigmentation within infected stem tissues provides a reliable visual diagnostic feature that distinguishes *V. longisporum* infection from other vascular diseases of *B. napus* (Eynck et al., 2007). Upon host senescence and tissue decomposition, microsclerotia are released into the soil, where they can persist as viable propagules for extended periods reported to survive for up to eight years under field conditions (Sarenqimuge et al., 2025) enabling pathogen carry-over between cropping seasons and re-infection of susceptible hosts in subsequent growing seasons (Eynck et al., 2007; Heale & Karapapa, 1999). Variation in symptom severity among cultivars further indicates that host genetic factors play an important role in determining disease outcomes and resistance levels (Eynck et al., 2009).

Unlike many soil-borne pathogens that primarily damage root tissues, *V. longisporum* establishes a vascular lifestyle following root penetration and subsequently colonizes xylem vessels, enabling systemic movement throughout the host plant (Eynck et al., 2007). At the molecular level, *B. napus* gene *BnHva22c*, which encodes a stress-responsive protein involved in intracellular vesicle trafficking, has been identified as a susceptibility factor that facilitates *V. longisporum* infection. Plants in which *BnHva22c* was knocked out using CRISPR showed significantly reduced pathogen colonization and disease severity, indicating that *V. longisporum* exploits this host gene to establish successful infection (Ye et al., 2024). Studies in *Arabidopsis thaliana* have further shown that systemic *V. longisporum* infection alters protein composition, metabolite profiles, and cell wall properties in the leaf apoplast, suggesting that vascular colonization triggers biochemical changes in tissues well beyond the original site of infection (Floerl et al., 2012).

The agricultural significance of *Verticillium* stem striping is amplified by the limited effectiveness of available management strategies. Because microsclerotia can survive in soil for many years and the pathogen remains protected within host vascular tissues during infection, chemical control measures often provide inconsistent or inadequate disease suppression (Depotter et al., 2016; Sarenqimuge et al., 2025). Consequently, disease management relies primarily on crop rotation, sanitation practices, and the use of resistant or tolerant cultivars when available (Klosterman et al., 2009). However, the long-term persistence of microsclerotia frequently limits the effectiveness of these approaches and complicates disease eradication from infested fields (Depotter et al., 2016; Sarenqimuge et al., 2025).

Although *Verticillium* stem striping was initially considered a disease of European *B. napus* production systems, the pathogen was subsequently confirmed in North American *B. napus*-growing regions, including Western Canada (Canadian Food Inspection Agency, 2015). The establishment of this pathogen within Canadian production areas is of particular concern because *B. napus* represents one of the country's most economically important field crops (Canola Council of Canada, 2024). Given the persistence of the pathogen in agricultural soils, the difficulty of controlling vascular infections, and the limited effectiveness of current management options, improving host resistance remains one of the most promising long-term strategies for disease management (Eynck et al., 2009; Ye et al., 2024). Understanding the defence mechanisms that restrict pathogen colonization and disease development is therefore essential for the development of durable resistance in *B. napus* and other *Brassica* crops.

2.3 Plant Defence Responses During *Verticillium longisporum* Infection

Plants possess multilayered defence systems that protect against fungal invasion through the coordinated action of structural barriers, immune recognition mechanisms, defence signalling networks, and antimicrobial responses (Bednarek et al., 2009; Jones & Dangl, 2006). Upon pathogen attack, plants rapidly perceive invading microorganisms and activate local and systemic defence responses that function to restrict pathogen colonization and limit disease development (Jones & Dangl, 2006; Liu et al., 2026). These defence mechanisms are particularly important in interactions involving *V. longisporum* that enters through the roots and subsequently colonizes xylem tissues, enabling systemic spread throughout the host plant (Eynck et al., 2007).

Consequently, disease development is largely determined by the ability of the host to restrict fungal penetration, vascular colonization, and pathogen movement within infected tissues (Eynck et al., 2007; Ye et al., 2024). The following sections describe the major structural, chemical, and signalling-based defence mechanisms that contribute to plant resistance against fungal pathogens.

2.3.1 Structural Defences and Cell Wall Reinforcement

Structural barriers constitute the first line of defence against fungal pathogens and play a critical role in preventing pathogen entry and colonization (Malinovsky et al., 2014; Underwood, 2012). The plant cell wall constitutes a preformed physical barrier that limits pathogen access and is actively remodeled and reinforced at sites of pathogen detection, representing the primary structural defence against microbial invasion (Underwood, 2012). Beyond their structural role, plant cell walls function as dynamic defence interfaces that participate in pathogen perception, immune signalling, and the activation of downstream defence responses (Malinovsky et al., 2014; Munzert & Engelsdorf, 2025). Plant cell walls are composed primarily of cellulose, hemicellulose, pectin and lignin and their composition can be actively modified following pathogen attack to enhance resistance (Malinovsky et al., 2014).

Successful fungal infection often requires the degradation or circumvention of these structural barriers. Many fungal pathogens including vascular wilt pathogens such as *V. longisporum* secrete cell wall-degrading enzymes, including cellulases, pectinases, and xylanases, which facilitate host penetration and tissue colonization (Klosterman et al., 2011; Munzert & Engelsdorf, 2025). In response to pathogen recognition and subsequent immune activation, plants rapidly reinforce cell walls through the deposition of defensive polymers and the activation of localized defence structures that restrict pathogen ingress (Underwood, 2012). One of the earliest structural responses is the formation of papillae at attempted penetration sites, structures enriched in callose and other antimicrobial compounds that strengthen the cell wall and impede pathogen entry (Voigt, 2014). In *B. napus*, CRISPR-mediated knockout of *BnaLYK*, a gene encoding a *LysM-domain receptor kinase* involved in chitin perception compromised pathogen detection responses and callose deposition, demonstrating that receptor-mediated chitin recognition is a key trigger of structural reinforcement responses in this crop (Liu et al., 2026).

Lignification represents another important component of structural defence. The deposition of lignin increases cell wall rigidity, reduces susceptibility to fungal cell wall-degrading enzymes,

and restricts pathogen movement through host tissues (Barros et al., 2015). Enhanced lignification has frequently been associated with increased resistance to vascular pathogens because it limits pathogen spread and reinforces host tissues surrounding infection sites (Miedes et al., 2014). In interactions involving *V. longisporum*, histological analyses have demonstrated increased lignification within vascular tissues following infection, and these responses have been associated with reduced fungal colonization and delayed disease development (Kashyap et al., 2026).

In addition to reinforcing cell walls, plants may restrict pathogen movement through the formation of vascular occlusions. The deposition of gels, gums, and related materials within xylem vessels can impede pathogen spread through the vascular system and limit systemic colonization in *Verticillium*-infected hosts (Fradin & Thomma, 2006). Although these responses may reduce fungal movement, excessive vascular occlusion can also interfere with water transport and contribute to disease symptoms characteristic of vascular wilt pathogens (Fradin & Thomma, 2006).

Studies have further showed that structural defence responses contribute directly to immune signalling. During pathogen attack, degradation of cell wall components can release damage-associated molecular patterns (DAMPs) which are endogenous signals functionally analogous to PAMPs that are recognised by host receptors and activate downstream defence pathways (Malinovsky et al., 2014; Munzert & Engelsdorf, 2025). Consequently, the cell wall functions not only as a passive barrier but also as an active participant in pathogen detection and defence regulation. The effectiveness of structural defence therefore depends on the balance between pathogen-mediated degradation of host tissues and the ability of the plant to reinforce and remodel structural barriers during infection (Fradin & Thomma, 2006; Munzert & Engelsdorf, 2025).

2.3.2 Chemical and Hormonal Defence Responses

In addition to structural barriers, plants deploy a diverse array of chemical and biochemical defence mechanisms that restrict pathogen growth and contribute to disease resistance (Haghpanah et al., 2025; Pieterse et al., 2012). These responses include the production of antimicrobial metabolites, reactive oxygen species (ROS), and defence-associated phytohormones that collectively enhance resistance against invading pathogens (Haghpanah et al., 2025; Pieterse et al., 2012).

Among the chemical defence mechanisms present in Brassicaceae species, the glucosinolate-myrosinase system plays a central role in resistance against microbial pathogens. Following tissue

damage or pathogen recognition, glucosinolates are hydrolysed by myrosinase enzymes to produce a range of biologically active compounds, including isothiocyanates and nitriles, many of which possess antimicrobial activity (Wittstock & Halkier, 2002). The importance of these metabolites in antifungal defence was highlighted by Bednarek et al. (2009), who showed that *A. thaliana* mutants impaired in indolic glucosinolate and camalexin biosynthesis exhibited significantly increased susceptibility to fungal infection, whereas disruption of camalexin biosynthesis alone was insufficient to alter resistance, indicating that these pathways act redundantly rather than independently in antifungal defence. Consistent with the role of glucosinolates in antifungal defence, infection by *V. longisporum* has been shown to alter both glucosinolate accumulation and glucosinolate breakdown-product profiles in Brassicaceae species, indicating that these defence metabolites are actively modulated during vascular colonization (Witzel et al., 2015). Together, these studies indicate that glucosinolate-derived metabolites contribute to antifungal defence and represent an important component of the host response against vascular pathogens.

Reactive oxygen species constitute another important component of plant chemical defence against pathogen invasion. Following pathogen recognition, membrane-bound NADPH oxidases generate a rapid oxidative burst that serves as one of the earliest immune responses activated during infection (Zhang et al., 2026). The accumulation of ROS at infection sites contributes directly to plant defence by restricting pathogen growth and reinforcing cell wall barriers through peroxidase-mediated oxidative cross-linking of cell wall proteins and the deposition of lignin and suberin (Haghpanah et al., 2025). In addition to their antimicrobial effects, ROS act as important signalling molecules that coordinate defence responses through interactions with salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) mediated pathways and are associated with hypersensitive cell death responses at infection sites during effector-triggered immunity (Jones & Dangl, 2006). Because excessive ROS accumulation can damage cellular components, plants maintain redox homeostasis through antioxidant systems involving enzymes such as superoxide dismutases, catalases, and ascorbate peroxidases, which regulate ROS levels and prevent oxidative damage while allowing ROS-mediated signalling to occur (Noctor et al., 2014). Together, these mechanisms enable ROS to function both as antimicrobial agents and as key regulators of plant defence signalling.

Phytohormones also function as central regulators of plant immunity, coordinating and amplifying defence responses following pathogen recognition. Among these, SA, JA, and ET constitute the principal hormonal signalling pathways involved in plant defence and operate through interconnected regulatory networks that are adjusted according to the lifestyle of the invading pathogen (Pieterse et al., 2012). SA is typically associated with defence against biotrophic and hemibiotrophic pathogens and promotes immune activation through NPR1, which interacts with TGA transcription factors to induce the expression of pathogenesis-related (PR) genes and establish systemic acquired resistance (Fang et al., 2025). Direct evidence for the involvement of SA during *V. longisporum* infection was provided by Ratzinger et al. (2009), who detected significantly increased concentrations of both SA and its glucoside conjugate in the xylem sap of infected *B. napus* plants, demonstrating that SA accumulates within vascular tissues during pathogen colonization. In contrast, JA and ET function cooperatively to regulate defence responses against necrotrophic pathogens and herbivorous insects, primarily through the COI1–JAZ–MYC2 signalling pathway (Pieterse et al., 2012). Extensive crosstalk between SA- and JA-dependent pathways enables plants to fine-tune and prioritize appropriate defence strategies while balancing competing physiological demands (Haghpanah et al., 2025). During plant-pathogen interactions, significant alterations in SA- and JA-associated metabolites were detected in the leaf apoplast of *A. thaliana* during *V. longisporum* infection, indicating that defence-related hormonal pathways are actively modulated during vascular pathogen colonization (Floerl et al., 2012). In addition to these defence-associated hormones, abscisic acid (ABA) plays an important role in integrating biotic and abiotic stress responses. ABA regulates stomatal aperture and water status and can modulate both SA- and JA-mediated signalling pathways, thereby influencing defence outcomes under conditions of simultaneous abiotic and biotic stress (Cutler et al., 2010). The interaction between defence-related and stress-responsive hormonal pathways is particularly relevant under combined stress conditions and therefore provides important context for understanding plant responses to simultaneous drought and pathogen challenge.

Together, glucosinolate-derived metabolites, ROS, and phytohormone signalling pathways form an integrated chemical defence network that restricts pathogen growth and coordinates downstream immune responses.

2.3.3 Immune Recognition and Defence Signalling

Successful defence against fungal pathogens depends on the ability of plants to rapidly recognise invading microorganisms and activate appropriate immune responses. Plant immunity is generally organized into two interconnected layers: pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones & Dangl, 2006). PTI is initiated when pattern recognition receptors (PRRs) located at the cell surface detect conserved pathogen-associated molecular patterns (PAMPs), such as fungal chitin and β -glucans (Jones & Dangl, 2006; Liu et al., 2026). Recognition of these molecules activates a range of defence responses, including ROS production, callose deposition, and the induction of defence-related genes (Liu et al., 2026). In *B. napus*, the LysM receptor kinase *BnaLYK* contributes to antifungal resistance by regulating ROS production, callose deposition, and defence-related gene expression following chitin perception, providing direct evidence that receptor-mediated recognition systems play an important role in *Brassica* immunity (Liu et al., 2026).

To overcome host defences, many fungal pathogens also secrete effector proteins that suppress PTI and facilitate colonization. Plants have evolved intracellular resistance proteins capable of recognizing these effectors either directly or indirectly, leading to the activation of ETI (Dodds & Rathjen, 2010). ETI is frequently associated with a stronger and more sustained defence response than PTI and may involve localized programmed cell death known as the hypersensitive response (HR), which restricts pathogen growth at infection sites (Dodds & Rathjen, 2010). Although originally described as distinct pathways, PTI and ETI were proposed by Jones and Dangl (2006) to function as interconnected components of a coordinated plant immune system that collectively contribute to disease resistance.

Activation of PTI and ETI initiates intracellular signalling networks involving calcium influx, ROS production, mitogen-activated protein kinase (MAPK) cascades, and extensive transcriptional reprogramming that collectively coordinate defence gene expression (Haghpanah et al., 2025; Jones & Dangl, 2006). Among these regulators, WRKY transcription factors play particularly important roles in integrating defence signals and modulating the expression of genes associated with pathogen resistance (Eulgem & Somssich, 2007). In Brassicaceae species, MYB transcription factors have been implicated in resistance to vascular pathogens. For example, the R2R3-MYB transcription factor BrMYB108 positively regulates resistance to *V. longisporum* in

B. rapa by directly activating respiratory burst oxidase homolog (Rboh) genes, thereby enhancing ROS production and restricting pathogen colonization (Su et al., 2023). Collectively, these signalling networks integrate pathogen perception with downstream defence responses, enabling plants to restrict pathogen colonization and disease development (Haghpanah et al., 2025; Jones & Dangl, 2006).

The complexity of transcriptional reprogramming during *B. napus*-*V. longisporum* interactions was further exhibited by Shen et al. (2014), who identified 62 miRNAs that responded to pathogen infection in *B. napus* roots. Among these, suppression of *miR168* was associated with increased expression of ARGONAUTE1 (AGO1), a central component of the RNA-induced silencing complex involved in PTI responses, whereas induction of *miR1885* promoted cleavage of transcripts encoding TIR-NBS-LRR resistance proteins (Shen et al., 2014). Functional analyses further showed that *ago1* mutants exhibited reduced *V. longisporum* colonization and disease symptoms compared with wild-type plants, indicating that miRNA-mediated regulatory pathways influence disease development during infection (Shen et al., 2014). Taken together, these findings suggest that post-transcriptional gene regulation contributes to the outcome of *B. napus*-*V. longisporum* interactions and highlights the importance of small RNA-mediated defence signalling during vascular pathogen infection. Although these defence mechanisms contribute to restricting *V. longisporum* colonization, disease development is not determined solely by host resistance and pathogen virulence. Environmental conditions strongly influence both plant physiology and pathogen behaviour and can therefore alter the outcome of infection (Kashyap et al., 2026). Among these environmental factors, drought is particularly important because it affects crop productivity worldwide and has the potential to modify host-pathogen interactions through changes in plant growth, water relations, and defence signalling (Elferjani & Soolanayakanahally, 2018; Lawlor & Cornic, 2002).

2.4 Drought Stress Responses in *Brassica napus*

Drought stress is one of the most significant abiotic constraints limiting agricultural productivity worldwide and is expected to increase in frequency and severity under future climate scenarios (IPCC, 2021). Among major field crops, *B. napus* is particularly vulnerable to water limitation, with drought reducing photosynthetic carbon assimilation, growth, and seed yield (Elferjani &

Soolanayakanahally, 2018). The vulnerability of *B. napus* to drought was starkly illustrated by the 2021 drought in Western Canada, which reduced national canola production by 35.4% to 12.6 million tonnes, resulting in the lowest national canola yield recorded since 2007 (Statistics Canada, 2021). This substantial yield reduction highlights the importance of understanding the physiological and molecular mechanisms underlying drought tolerance in *B. napus*. The following sections describe the major physiological, morphological, anatomical, and molecular responses to drought stress in *B. napus* that provide the biological context necessary for understanding how water deficit modifies plant-pathogen interactions under combined stress conditions.

2.4.1 Effect of Drought Stress on Growth and Development

Drought stress reduces soil water availability and disrupts plant water relations, resulting in decreased leaf water potential, reduced cellular turgor, and impaired growth (Lawlor & Cornic, 2002). One of the earliest physiological responses to water deficit is stomatal closure, which attenuates transpirational water loss and helps maintain plant water status (Lawlor & Cornic, 2002). In *B. napus*, regulation of stomatal conductance plays a critical role in drought adaptation and reduced stomatal conductance has been associated with slower water loss and improved survival under drought stress, without a corresponding reduction in net photosynthetic rate (Wu et al., 2026). However, the efficiency of this trade-off is genotype-dependent, comparative evaluation of drought-tolerant and drought-sensitive *B. napus* genotypes revealed that drought stress significantly reduces photosynthetic performance, with the magnitude of reduction varying substantially among genotypes (Bouchyoua et al., 2026). Beyond genotype, as water limitation becomes more severe, chlorophyll degradation, disruption of photosystem function, and reduced carbon fixation capacity further impair photosynthetic efficiency (Amiri & Hemmati Hassan Gavyar, 2026). This decline in photosynthetic performance is accompanied by reduced biomass accumulation, lower seed yield, and changes in seed quality traits in *B. napus* (Elferjani & Soolanayakanahally, 2018). Water limitation during flowering and seed filling is particularly detrimental because these developmental stages largely determine final yield and oil production (Ghobadi et al., 2006). Consequently, drought stress during reproductive development can substantially reduce pod number per plant, seed weight, and overall crop productivity (Ghobadi et al., 2006).

Drought stress also disrupts cellular redox homeostasis through the excessive accumulation of ROS, including superoxide radicals and hydrogen peroxide (Miller et al., 2010). Elevated ROS levels can damage proteins, membrane lipids, and nucleic acids, resulting in oxidative stress and impaired cellular function (Mittler, 2002). To counteract oxidative damage, drought-stressed *B. napus* plants activate antioxidant defence systems, including superoxide dismutase and peroxidase, which contribute to ROS detoxification and maintenance of cellular homeostasis (Abedi & Pakniyat, 2010). Enhanced antioxidant activity has been associated with improved drought tolerance in *B. napus* through the maintenance of photosynthetic activity and reduction of oxidative injury (Ayyaz et al., 2021).

2.4.2 Morphological and Anatomical Adaptations to Drought Stress

Drought stress induces morphological modifications that influence water acquisition, reduce water loss, and improve plant survival under water-limited conditions (Bouchyoua et al., 2026). These responses involve coordinated changes in root architecture, biomass allocation, leaf morphology, vascular organization, and the development of root protective barriers (Amesalu & Kebede, 2020).

Root system architecture plays a central role in determining the capacity of plants to acquire soil resources under drought conditions. Deep-rooted phenotypes generally enhance access to water reserves located in deeper soil layers, whereas shallow root systems are often more effective for phosphorus acquisition, reflecting a tradeoff between water and nutrient foraging strategies (Lynch, 2011). Genome-wide analyses of *B. napus* germplasm have revealed substantial variation in root length, root diameter, root surface area, and lateral root development, indicating that root architectural traits represent an important source of adaptive variation within the species (Yang et al., 2024). Consistent with these observations, drought-tolerant *B. napus* genotypes maintain greater root length and root volume under water-deficit conditions, traits that are associated with improved drought performance (Bouchyoua et al., 2026). Similar evidence has been reported in rice, where the *DEEPER ROOTING 1 (DRO1)* gene promotes steeper root growth angles, thereby improving access to deep soil moisture and enhancing drought avoidance and grain yield under field conditions without increasing cellular dehydration tolerance (Uga et al., 2013). Together, these studies show that root architectural traits and biomass allocation patterns are important determinants of drought adaptation across crop species (Bouchyoua et al., 2026; Uga et al., 2013; Yang et al., 2024). Water limitation also affects shoot morphology. In *B. napus*, drought stress

reduces leaf development and leaf area, reflecting impaired plant water status and contributing to a reduction in the transpiring surface (Sepehri & Golparvar, 2011). In addition, combined drought and heat stress alter cuticular wax composition and accumulation, suggesting that modifications of the cuticle may help reduce non-stomatal water loss under adverse environmental conditions (Tomasi et al., 2024).

Beyond changes in overall plant morphology, drought stress induces extensive anatomical remodelling within root tissues. Water limitation has been associated with alterations in root tissue organization, modifications of vascular structure, and changes in root growth patterns that facilitate access to soil water resources (Kashyap et al., 2026; Yang et al., 2024). Drought conditions also trigger substantial remodelling of root cell walls, including changes in callose deposition, pectin distribution, and fibre formation, which strengthen cell walls and contribute to the maintenance of tissue integrity under stress conditions (Diana et al., 2024). Together, these structural adjustments highlight the importance of root anatomical plasticity in maintaining plant function during water deficit.

Recent work by Kashyap et al. (2026) further highlighted that severe drought stress promotes extensive anatomical modifications in both roots and hypocotyls of *B. napus*. Drought-treated plants exhibited increased suberization and the formation of protective suberized tissues, indicating that structural reinforcement is an important component of adaptation to prolonged water limitation (Kashyap et al., 2026). The study also reported changes in tissue organization and barrier formation, suggesting that drought-induced structural plasticity may help sustain root function under adverse environmental conditions.

The enhanced suberization observed under drought is particularly important because suberin is a major structural component of root apoplastic barriers that regulate the radial movement of water and solutes. Apoplastic barriers formed within the endodermis restrict diffusion across root tissues and contribute to the regulation of water and nutrient transport (Naseer et al., 2012). The development and reinforcement of these barriers can regulate water and nutrient transport under environmental stress conditions (Kim et al., 2022). Genetic studies in tomato have shown that the MYB transcription factor MYB92 is required for exodermal suberin deposition, and that loss of MYB92 function significantly impairs plant water status including reduced stem water potential, transpiration, and stomatal conductance under water-limited conditions (Cantó-Pastor et al., 2024).

Therefore, these findings indicate that root suberization is a functionally important, evolutionarily conserved adaptation that directly contributes to plant performance under water deficit.

Taken together, these studies showed that drought adaptation in *B. napus* depends not only on modifications of root architecture and biomass allocation but also on extensive anatomical remodelling involving vascular tissues, cell-wall reinforcement, suberization, and the formation of apoplastic barriers that help maintain water transport and tissue function under water-limited conditions.

2.4.3 Molecular Regulation of Drought Responses

Drought stress triggers molecular signalling pathways that enable plants to perceive water deficit and activate stress-responsive gene expression (Yamaguchi-Shinozaki & Shinozaki, 1993). Among these responses, ABA signalling functions as a central regulatory pathway coordinating physiological and transcriptional responses to water limitation (Fujita et al., 2005). Early evidence for ABA-mediated drought regulation was provided through the characterization of the drought-responsive gene *RD29A*, whose expression is strongly induced by both dehydration and ABA treatment (Yamaguchi-Shinozaki & Shinozaki, 1993). Subsequent analyses identified ABA-responsive cis-acting elements within the promoters of drought-inducible genes, demonstrating that ABA signalling directly regulates stress-responsive transcriptional networks (Yamaguchi-Shinozaki & Shinozaki, 1994).

Perception of ABA is mediated by members of the PYRABACTIN RESISTANCE/PYR1-LIKE (PYR/PYL) receptor family, which initiate intracellular signalling through inhibition of type 2C protein phosphatases (PP2Cs) (Park et al., 2009). Subsequent studies established that subclass III SnRK2 kinases are essential positive regulators of ABA signalling, phosphorylating ABF/AREB transcription factors and promoting ABA-responsive gene expression (Fujii & Zhu, 2009). This ABA signalling model further showed that ABA-bound PYR/PYL/RCAR receptors inhibit PP2Cs, thereby releasing SnRK2s from negative regulation and enabling activation of downstream ABA-responsive transcriptional programs (Fujii et al., 2009; Umezawa et al., 2010). Functional analyses also revealed that members of the ABRE-binding factor (ABF/AREB) family act as major transcriptional regulators of ABA-dependent drought responses (Fujita et al., 2005). Overexpression of *AREB1* enhanced drought tolerance and induced the expression of numerous

ABA-responsive genes, highlighting its important role in ABA-mediated adaptation to water deficit conditions (Fujita et al., 2005).

In addition to regulating transcriptional responses, ABA signalling directly controls stomatal behaviour and transpiration, thereby influencing plant water status during drought stress (Mega et al., 2019). Genetic manipulation of ABA signalling components in wheat altered transpiration and water-use efficiency while improving performance under water-deficit conditions, underscoring ABA-mediated regulation of water use in crop drought adaptation (Mega et al., 2019). Beyond ABA signalling, drought-responsive gene expression is extensively regulated by transcription factors that coordinate downstream stress-response pathways. Members of the Dehydration Responsive Element Binding (DREB) family bind dehydration-responsive elements (DREs) in the promoters of target genes and regulate drought-responsive gene expression through ABA-independent signalling pathways, thereby contributing to abiotic stress tolerance (Liu et al., 1998; Sakuma et al., 2006). NAC transcription factors contribute to drought adaptation by regulating stress-responsive gene expression and coordinating regulatory networks associated with abiotic stress tolerance (Nakashima et al., 2012). WRKY proteins participate in the integration of abiotic stress and defence signalling networks and have been implicated in the regulation of drought-responsive pathways (Rushton et al., 2010). For example, the guard-cell-specific transcription factor AtMYB60 regulates stomatal opening and water loss, demonstrating an important role for MYB-mediated regulation in plant drought responses (Cominelli et al., 2005). Collectively, these transcription factor families coordinate extensive transcriptional reprogramming that regulates downstream stress-response pathways and contributes to plant adaptation under drought conditions (Nakashima et al., 2012; Sakuma et al., 2006).

Evidence from *B. napus* further supports the importance of transcriptional regulation in drought adaptation. Comparative transcriptomic analyses of drought-stressed roots and leaves identified numerous drought-responsive genes associated with ABA signalling, detoxification processes, transcriptional regulation, and osmotic stress responses (Liu et al., 2015). Integrated physiological and transcriptomic investigations further revealed substantial changes in stress-responsive gene expression and osmotic adjustment mechanisms, pointing to coordinated molecular responses during drought adaptation in *B. napus* (Wang et al., 2019). More recently, functional characterization of *BnaMYB52* revealed that this MYB transcription factor negatively regulates

drought resistance by controlling both stomatal and non-stomatal water loss, providing direct evidence that MYB-mediated regulatory networks contribute to drought adaptation in *B. napus* (Wu et al., 2026). These studies demonstrate that drought adaptation in *B. napus* involves coordinated regulation of ABA signalling, stress-responsive gene expression, and physiological processes associated with water conservation under drought conditions (Liu et al., 2015; Wang et al., 2019; Wu et al., 2026).

Despite considerable progress in identifying drought-responsive genes and regulatory pathways in *B. napus*, less is known about how these responses are coordinated when plants are exposed to multiple environmental stresses simultaneously (Atkinson & Urwin, 2012; Ramegowda & Senthil-Kumar, 2015). Drought stress influences multiple biological processes, including hormone signalling, transcriptional regulation, defence responses, and vascular development, all of which also contribute to plant interactions with vascular pathogens (Kashyap et al., 2026; Rushton et al., 2010). Consequently, simultaneous exposure to drought stress and *V. longisporum* may generate unique physiological, anatomical, and molecular responses that cannot be predicted from either stress alone, highlighting the importance of investigating combined stress interactions in *B. napus* (Atkinson & Urwin, 2012; Ramegowda & Senthil-Kumar, 2015).

2.5 Interaction Between Drought Stress and *Verticillium longisporum* Infection

Drought stress and *V. longisporum* infection each independently induce substantial physiological and structural changes in *B. napus* (Eynck et al., 2007; Lawlor & Cornic, 2002). However, plants in natural and agricultural environments are frequently exposed to multiple stresses simultaneously and combined abiotic and biotic stresses often elicit unique physiological, molecular, and transcriptional responses that cannot be predicted from individual stress responses alone (Ramegowda & Senthil-Kumar, 2015). Understanding how drought stress modifies the *B. napus* - *V. longisporum* interaction is therefore essential for predicting disease outcomes and for identifying the molecular mechanisms that may underlie altered host susceptibility or resistance during combined stress.

2.5.1 Effects of Drought Stress on Plant-Pathogen Interactions

Drought stress is widely recognised as an important environmental factor influencing plant-pathogen interactions because water limitation alters plant physiology, metabolism, and defence

signalling, thereby affecting host susceptibility and resistance responses (Ramegowda & Senthil-Kumar, 2015; Rivero et al., 2022). However, the effects of drought on disease development are highly context dependent and vary according to the host species, pathogen lifestyle, severity of water limitation, and timing of stress exposure (Atkinson & Urwin, 2012; Ramegowda & Senthil-Kumar, 2015; Rivero et al., 2022). Several studies have shown that prior drought acclimation can enhance plant resistance to subsequent pathogen infection. Ramegowda et al. (2013) reported that drought-acclimated *Nicotiana benthamiana* plants exhibited reduced disease severity following infection by *Sclerotinia sclerotiorum* and *Pseudomonas syringae* pv. *tabaci*. Similarly, Martínez-Ferri et al. (2019) demonstrated that mild water-stress priming enhanced tolerance to *Rosellinia necatrix* in susceptible avocado rootstocks, resulting in delayed disease progression and symptom development. Barradas et al. (2018) showed that drought priming slightly reduced susceptibility of *Eucalyptus globulus* to *Neofusicoccum eucalyptorum* compared with non-primed plants. Together, these studies indicate that drought priming can alter plant physiological and molecular responses in ways that contribute to enhanced tolerance or reduced susceptibility to subsequent pathogen infection (Barradas et al., 2018; Martínez-Ferri et al., 2019; Ramegowda et al., 2013).

Other studies, however, show that drought stress can increase disease susceptibility by promoting pathogen colonization and weakening host defence responses. Irulappan et al. (2022) showed that drought accelerated root colonization by *Macrophomina phaseolina*, facilitated fungal spread beyond the endodermal barrier into vascular tissues, and this was associated with downregulation of innate immune and defence-related genes. Similarly, Sinha et al. (2019) reported that combined drought and pathogen stress negatively affected chickpea performance under field conditions, resulting in reduced plant growth and significant yield losses compared with well-watered and pathogen-only treatments. These observations indicate that drought may increase disease severity and, in some cases, compromise host defence under specific environmental and biological conditions (Irulappan et al., 2022; Sinha et al., 2019).

The contrasting outcomes reported across different pathosystems suggest that drought-induced resistance or susceptibility depends on complex interactions among plant physiology, pathogen biology, hormonal regulation, and stress timing (Ramegowda & Senthil-Kumar, 2015; Rivero et al., 2022). Consequently, direct experimental studies are required to determine how drought influences disease development in individual host-pathogen systems.

2.5.2 Evidence for Interaction Between Drought Stress and *Verticillium longisporum*

Direct experimental evidence for the interaction between drought stress and *V. longisporum* infection in *B. napus* was provided by Kashyap et al. (2026). The study highlighted that severe drought (30% field capacity) reduced the acropetal movement of *V. longisporum* from roots to hypocotyls and was associated with reduced disease severity. Transcriptomic and histological analyses further revealed enhanced suberin deposition and the formation of apoplastic barriers in root tissues under drought conditions, suggesting that drought-induced structural modifications restricted pathogen movement through the vascular system (Kashyap et al., 2026). These findings identified a potential mechanistic link between drought-induced anatomical changes and reduced pathogen colonization and highlighted the importance of cell wall-associated defence responses during combined stress.

In contrast, Lopisso et al. (2017) reported that drought stress did not significantly alter fungal colonization or disease severity during *V. longisporum* infection in winter oilseed rape. Based on measurements of fungal biomass, physiological parameters, and drought-responsive gene expression, the authors concluded that drought stress and pathogen infection influenced host physiology through largely independent mechanisms under the experimental conditions examined (Lopisso et al., 2017). The contrasting observations reported by Kashyap et al. (2026) and Lopisso et al. (2017) indicate that the outcome of drought-*V. longisporum* interactions is influenced by factors such as stress intensity and duration, experimental conditions, host responses, and the timing of stress exposure.

The molecular mechanisms underlying these responses remain poorly understood. One possibility is that drought alters the regulatory state of the plant and thereby influences subsequent responses to pathogen infection. Stress-induced changes in gene expression can be mediated through epigenetic mechanisms, including DNA methylation, histone modifications, and chromatin remodelling, which regulate gene activity without altering the underlying DNA sequence (Crisp et al., 2016; Lämke & Bäurle, 2017; Zhang et al., 2018). Among these mechanisms, DNA methylation has received particular attention because of its widespread occurrence throughout plant genomes and its role in regulating gene expression, genome stability, and stress-responsive pathways. Understanding the function of DNA methylation during plant

stress responses may therefore provide insights into the mechanisms through which drought modifies host-pathogen interactions.

2.6 Epigenetic Regulation in Plants

One possible mechanism linking drought to changes in pathogen colonization is epigenetic regulation, particularly DNA methylation. While most stress-responsive changes in gene expression are driven by signalling pathways and transcription factors (Kilian et al., 2007), epigenetic mechanisms are also known to contribute to the regulation of stress-responsive genes (Miryeganeh, 2021; Zhang et al., 2018). Epigenetic regulation refers to modifications in gene activity that occur without alterations in the underlying DNA sequence and are mediated through molecular processes that influence chromatin structure and gene accessibility (Bird, 2007; Law & Jacobsen, 2010). Among the various epigenetic mechanisms identified in plants, DNA methylation is one of the most extensively studied because of its widespread occurrence throughout plant genomes and its ability to influence gene expression, transposable element (TE) activity, and genome stability (Law & Jacobsen, 2010; Zhang et al., 2018).

2.6.1 DNA Methylation in Plants

DNA methylation involves the addition of a methyl group to the fifth carbon position of cytosine residues, resulting in the formation of 5-methylcytosine (Gehring & Henikoff, 2008). Unlike mammals, where DNA methylation occurs predominantly in CG dinucleotide contexts, plant genomes exhibit methylation in three distinct sequence contexts: CG, CHG, and CHH, where H represents adenine, cytosine, or thymine (Lister et al., 2008). The establishment and maintenance of DNA methylation patterns are mediated by several classes of DNA methyltransferases. De novo methylation is primarily catalyzed by DOMAINS REARRANGED METHYLTRANSFERASE 2 (*DRM2*) through the RNA-directed DNA methylation (RdDM) pathway, a process that uses small interfering RNAs to guide methylation to specific genomic loci (Cao & Jacobsen, 2002; Zhong et al., 2014). Maintenance of CG and CHG methylation during DNA replication is achieved through the action of METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3), respectively (Bartee & Bender, 2001), while CHH methylation is maintained through the activity of CMT2 and the RdDM pathway (Law & Jacobsen, 2010). Together, these enzymes ensure the transmission of methylation patterns during cell division and contribute to epigenetic stability

throughout plant development (Gehring & Henikoff, 2008). DNA methylation patterns are not solely established through methyltransferase activity but are also regulated through active DNA demethylation mechanisms. In plants, DNA glycosylases such as REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DEMETER-LIKE 2 (DML2), and DEMETER-LIKE 3 (DML3) remove methylated cytosines through a base-excision repair pathway, thereby allowing dynamic regulation of methylation states in response to developmental and environmental cues (Zhu, 2009). This dynamic balance between DNA methylation and demethylation therefore enables plants to modify gene expression in response to developmental and environmental signals, thereby contributing to stress adaptation and phenotypic plasticity (Zhang et al., 2018).

2.6.2 Genomic Distribution and Function of DNA Methylation

DNA methylation is widely distributed throughout plant genomes and occurs in TEs, repetitive sequences, gene bodies, and regulatory regions (Lister et al., 2008). A major function of DNA methylation is the suppression of TE activity, thereby contributing to genome stability (Liang et al., 2022). Consistent with this role, Liang et al. (2022) reported that 45.6% of activated TEs in *A. thaliana* were associated primarily with the loss of CG methylation, whereas 34.4% were activated only when both CG and non-CG methylation pathways were disrupted, highlighting the contribution of multiple methylation pathways to TE silencing. In addition to transposon silencing, DNA methylation also influences gene regulation. Genome-wide analyses in rice demonstrated that promoter methylation is generally associated with reduced transcriptional activity, with promoter-unmethylated genes exhibiting significantly higher expression levels than promoter-methylated genes (Li et al., 2012). In contrast, gene body methylation is frequently associated with constitutively expressed housekeeping genes, although its precise biological functions remain incompletely understood (Bewick & Schmitz, 2017). These observations indicate that the effects of DNA methylation on gene expression vary according to genomic location.

Additionally, DNA methylation plays important roles in plant growth and development by regulating gene expression during key developmental transitions. Disruption of DNA methylation homeostasis has been shown to cause developmental abnormalities in *A. thaliana*, highlighting the importance of methylation-mediated regulation during plant development (Finnegan et al., 1996). DNA methylation is also involved in reproductive processes such as genome imprinting and endosperm development, where differential methylation of maternal and paternal genomes

contributes to parent-of-origin gene expression patterns (Gehring et al., 2009; Hsieh et al., 2009). Dynamic changes in DNA methylation have additionally been reported during seed development and germination, including increases in CHH methylation during seed maturation (Lin et al., 2017). Taken together, these findings support the idea that DNA methylation contributes to plant development, reproduction, and genome regulation.

2.6.3 DNA Methylation Responses to Abiotic Stress

Beyond its developmental functions, DNA methylation patterns can also be modified in response to abiotic stresses. Studies conducted across diverse plant species have reported changes in DNA methylation patterns under drought, salinity, heat, cold and heavy metal stress, indicating that DNA methylation occurs across a wide range of environmental conditions (Lodhi & Srivastava, 2025). In rice, drought stress induced both methylation and demethylation events throughout the genome, with the resulting methylation patterns differing between drought-tolerant and drought-sensitive genotypes. Approximately 70% of drought-induced methylation changes were reversed following recovery, whereas nearly 30% persisted after stress removal, suggesting that some methylation changes may contribute to stress memory (Wang et al., 2011). Similar responses have been reported in other plant species, although the magnitude and direction of methylation changes vary considerably. In *Medicago ruthenica*, drought stress reduced overall genomic methylation levels and was associated with differential methylation of genes involved in ABA and proline biosynthesis pathways (Zi et al., 2024). Hypomethylation of the *ABA2* and *P5CS* genes corresponded with increased gene expression, suggesting that drought-induced methylation changes may influence pathways involved in hormone signalling and osmotic adjustment during water deficit (Zi et al., 2024).

Abiotic stresses can also influence the expression of genes involved in DNA methylation and demethylation pathways. In *B. napus*, salt and heat stress altered the expression of several methyltransferase and demethylase genes, including members of the *MET*, *CMT*, *DRM*, *DNMT2*, and *ROS1* families (Fan et al., 2020), suggesting that environmental stress may affect the machinery responsible for methylation maintenance and establishment. Nutrient deprivation has also been linked to DNA methylation dynamics. Using whole-genome bisulfite sequencing, Secco et al. (2015) established that phosphate starvation induced differential methylation at discrete genomic regions located near stress-responsive genes and transposable elements. Unlike genome-

wide methylation shifts, these changes were concentrated at specific loci, suggesting that environmental stress can induce localized epigenetic modifications (Secco et al., 2015). Environmental variation has also been associated with site-specific DNA methylation differences in *Hevea brasiliensis*, where clone-specific and location-specific methylation patterns were detected within regulatory regions of genes involved in rubber biosynthesis and defence responses (Uthup et al., 2011).

Collectively, evidence from multiple plant species indicates that abiotic stresses can alter DNA methylation patterns, although the extent and direction of these changes differ among species, tissues, genotypes, and environmental conditions (Lodhi & Srivastava, 2025).

2.6.4 DNA Methylation Responses to Pathogen Infection

In addition to abiotic stresses, changes in DNA methylation patterns have also been reported during plant responses to pathogen infection. In *A. thaliana*, exposure to bacterial pathogens and SA induced numerous differentially methylated regions, many of which were associated with changes in gene expression. Stress-induced methylation changes frequently occurred within transposable elements and repetitive sequences and were linked to altered transcription of nearby genes, suggesting that dynamic DNA methylation contributes to the regulation of plant immune responses (Downen et al., 2012). In *Populus* (poplar), a disease-resistant genotype displayed lower basal DNA methylation levels and stronger pathogen-induced CHH hypomethylation than a susceptible genotype following *Lonsdalea populi* infection (Xiao et al., 2021). Consistent with the findings of Downen et al. (2012), these methylation changes were associated with defence-related genes and transcriptional responses to pathogen challenge.

The role of DNA methylation in plant-pathogen interactions is not restricted to the host, as fungal pathogens also exhibit dynamic methylation patterns associated with development and infection. In the rice blast fungus *Magnaporthe oryzae*, whole-genome bisulfite sequencing revealed that DNA methylation is concentrated within transposable element-rich regions and undergoes extensive reprogramming during fungal development. Methylation patterns differed markedly among mycelia, conidia, and appressoria, indicating that the fungal methylome is dynamically regulated throughout the infection cycle (Jeon et al., 2015). Furthermore, disruption of fungal DNA methyltransferases reduced conidial production and delayed appressorium

formation, suggesting that DNA methylation contributes to normal fungal development and the regulation of transposable element activity (Jeon et al., 2015).

The potential involvement of DNA methylation in pathogen adaptation and virulence has also been investigated in vascular pathogens. In *V. dahliae*, comparative methylation analyses of isolates displaying different levels of virulence revealed an inverse relationship between DNA methylation and pathogenicity, with more virulent isolates exhibiting lower methylation levels in numerous protein-coding genes associated with energy metabolism, membrane transport, sporulation, and host invasion processes (Ramírez-Tejero et al., 2020). Similarly, in the bacterial vascular pathogen *Ralstonia pseudosolanacearum*, differential DNA methylation sites emerged rapidly during experimental evolution on host plants and contributed to adaptation to the host environment, with some methylation changes remaining stable across multiple generations (Gopalan-Nair et al., 2024). Overall, these studies illustrate that DNA methylation participates in multiple aspects of plant-pathogen interactions, including the regulation of host immune responses, variation in disease resistance among genotypes, fungal development, and pathogen adaptation.

2.7 Approaches for DNA Methylation Analysis

Understanding DNA methylation requires analytical methods capable of identifying methylated cytosines at high resolution. Early approaches relied on sodium bisulfite conversion of DNA, whereas recent long-read sequencing technologies enable direct detection of DNA methylation from native DNA molecules (Frommer et al., 1992; Sigurpalsdottir et al., 2024).

2.7.1 Whole-Genome Bisulfite Sequencing

Bisulfite sequencing forms the basis of many modern DNA methylation analyses and has long been regarded as a gold-standard approach for determining cytosine methylation patterns at single-base resolution (Frommer et al., 1992; Meissner et al., 2005). Sodium bisulfite converts unmethylated cytosines to uracils while leaving 5-methylcytosines largely unaffected, enabling methylated and unmethylated cytosines to be distinguished following PCR amplification and sequencing (Frommer et al., 1992). To extend bisulfite sequencing beyond individual loci, Meissner et al. (2005) introduced reduced representation bisulfite sequencing (RRBS), a large-scale approach for high-resolution methylation analysis across representative portions of the genome. Genome-wide bisulfite sequencing was subsequently applied to generate single-base

resolution methylomes in *A. thaliana*, revealing methylation patterns across CG, CHG, and CHH contexts at genome-wide scale (Lister et al., 2008). Subsequent advances in sequencing technologies and bioinformatic pipelines further improved the analysis of bisulfite sequencing data and facilitated the identification of differentially methylated cytosines and regions across biological conditions (Adusumalli et al., 2014).

Despite its widespread use, bisulfite sequencing has several limitations. The method relies on the chemical conversion of unmethylated cytosines rather than direct detection of modified bases, requiring methylation status to be inferred following sequencing (Frommer et al., 1992). Efficient bisulfite treatment is critical because incomplete conversion of unmethylated cytosines can lead to false-positive methylation calls, whereas inappropriate conversion of methylated cytosines may result in underestimation of methylation levels (Genereux et al., 2008). In addition, the harsh chemical conditions used during bisulfite treatment can cause substantial DNA degradation and fragmentation, reducing DNA recovery and potentially introducing biases into downstream analyses (Grunau et al., 2001; Olova et al., 2018). Bisulfite conversion also reduces sequence complexity through the conversion of cytosines to thymines, which can complicate read alignment and downstream methylation analysis (Olova et al., 2018). Furthermore, conventional bisulfite sequencing cannot distinguish 5-methylcytosine from 5-hydroxymethylcytosine without additional chemical treatments such as oxidative bisulfite sequencing (Sigurpalsdottir et al., 2024). These limitations have motivated the development of alternative technologies capable of detecting DNA methylation directly from native DNA molecules.

2.7.2 Long-Read Sequencing Technologies for DNA Methylation Analysis

Advances in long-read sequencing technologies have enabled direct detection of DNA methylation from native DNA molecules without the need for bisulfite conversion (Gouil & Keniry, 2019). One such technology is Single-Molecule Real-Time (SMRT) sequencing developed by Pacific Biosciences (PacBio), which determines DNA sequence through real-time monitoring of nucleotide incorporation by DNA polymerase (Flusberg et al., 2010). Unlike bisulfite-based approaches, DNA modifications can be identified directly because modified bases alter polymerase kinetics during sequencing, generating characteristic changes in interpulse duration and other kinetic signals (Feng et al., 2013; Flusberg et al., 2010). The long-read capability of SMRT sequencing further facilitates the analysis of repetitive genomic regions and other complex

genomic features that are difficult to resolve using short-read technologies (Amarasinghe et al., 2020). More recently, improvements in PacBio circular consensus sequencing (CCS) and deep-learning-based methylation-calling methods have substantially increased methylation detection accuracy and enabled applications such as haplotype-aware methylation analysis (Ni et al., 2023).

Despite these advantages, methylation detection using PacBio relies on indirect inference from polymerase kinetic signals, and accurate identification of 5-methylcytosine modifications depends on the quality and depth of kinetic data obtained during sequencing (Feng et al., 2013; Flusberg et al., 2010). Therefore, the development of Oxford Nanopore Technologies (ONT) provided an alternative long-read sequencing platform capable of directly detecting DNA methylation from native DNA molecules (Gouil & Keniry, 2019). In ONT sequencing, single-stranded DNA molecules pass through biological nanopores while changes in ionic current are continuously measured, with modified and unmodified nucleotides generating distinct electrical signal patterns that can be used to identify DNA modifications (Wang et al., 2021). Unlike bisulfite sequencing, ONT does not require chemical conversion, PCR amplification, or additional laboratory treatments, allowing nucleotide sequence and methylation information to be obtained simultaneously from the same DNA molecule (Gouil & Keniry, 2019; Sigurpalsdottir et al., 2024). ONT read lengths are sufficient to resolve repetitive genomic regions, structural variation, haplotypes, and complex genomes that short-read technologies cannot adequately characterize (Amarasinghe et al., 2020). Improvements in nanopore chemistry, sequencing accuracy, and methylation-calling algorithms have further enhanced the utility of ONT for epigenomic research (Liu et al., 2021). Benchmarking studies have showed strong agreement between nanopore-derived methylation profiles and conventional bisulfite sequencing approaches, supporting the reliability of ONT for genome-wide DNA methylation analysis (Sigurpalsdottir et al., 2024). These advantages make ONT a useful platform for epigenetic studies in plants and other complex eukaryotic genomes (Gouil & Keniry, 2019).

2.8 Knowledge Gap, Hypothesis, and Research Objectives

Considerable progress has been made in understanding the physiological, molecular, and epigenetic responses of plants to environmental stress. Studies have exhibited that DNA methylation is dynamically regulated during both abiotic stress and pathogen infection and can influence the expression of stress-responsive genes, plant immunity, and adaptation to changing

environmental conditions (Dowen et al., 2012; Xiao et al., 2021; Zi et al., 2024). Furthermore, DNA methylation changes have been associated with drought adaptation, pathogen resistance, fungal development, and pathogen virulence, highlighting the importance of epigenetic regulation in both host and pathogen responses to stress (Gopalan-Nair et al., 2024; Jeon et al., 2015; Ramírez-Tejero et al., 2020).

Recent work by Kashyap et al. (2026) provided direct experimental evidence that drought stress substantially modifies the interaction between *B. napus* and *V. longisporum* at the physiological level. Specifically, drought reduced disease severity and restricted the acropetal movement of the pathogen from roots into the hypocotyl, demonstrating that water limitation can fundamentally alter pathogen colonization dynamics in *B. napus*. Despite this physiological characterization, the regulatory processes underlying reduced pathogen colonization under drought conditions remain entirely uncharacterized at the molecular level, and whether epigenetic changes, particularly genome-wide shifts in DNA methylation contribute to these altered host responses has not been investigated.

Although DNA methylation participates in plant responses to both abiotic and biotic stresses, most studies have examined these stressors independently rather than in combination (Dowen et al., 2012; Fan et al., 2020; Ramírez-Tejero et al., 2020; Wang et al., 2011; Zi et al., 2024). Consequently, the role of DNA methylation in regulating plant responses under simultaneous abiotic and biotic stress conditions remains poorly understood. This knowledge gap is particularly evident in crop species such as *B. napus*, where genome-wide investigations of DNA methylation dynamics under combined stress conditions are absent. Addressing this gap requires first establishing the individual epigenetic responses of *B. napus* to *V. longisporum* infection and drought stress independently, to provide a necessary baseline against which combined stress methylation dynamics can be evaluated and interpreted. Whether these individual and combined stress conditions induce distinct methylation responses, particularly at defence-related genes, hormone signalling loci, and genomic loci associated with pathogen recognition and immune regulation remains entirely unknown.

Characterizing genome-wide DNA methylation in *B. napus* presents particular technical challenges owing to the allotetraploid nature of this crop species, whose large and highly repetitive genome contains extensive homoeologous regions derived from its diploid progenitors *B. rapa* and

B. oleracea (Chalhoub et al., 2014). These features complicate short-read bisulfite sequencing approaches, which suffer from reduced mapping efficiency and ambiguous read assignment in repetitive and homoeologous regions (Olova et al., 2018). Advances in long-read sequencing technologies address these limitations by enabling direct detection of DNA methylation from native DNA molecules and producing reads sufficiently long to span and accurately assign repetitive genomic regions, complex structural features, and homoeologous loci (Amarasinghe et al., 2020; Gouil & Keniry, 2019; Sigurpalsdottir et al., 2024). ONT sequencing therefore represents a well-suited approach for genome-wide epigenetic profiling in complex polyploid genomes such as *B. napus* (Amarasinghe et al., 2020; Gouil & Keniry, 2019).

In total, the physiological evidence provided by Kashyap et al. (2026) and the literature on stress-induced epigenetic regulation highlight the need to investigate the genome-wide DNA methylation dynamics associated with *V. longisporum* infection, drought stress, and their combination in *B. napus*. Addressing this knowledge gap may improve understanding of the molecular mechanisms underlying the drought-modified host-pathogen interaction and facilitate the identification of candidate epigenetic markers associated with resilience to simultaneous environmental challenges.

Hypothesis

The simultaneous occurrence of drought stress and *V. longisporum* infection in *B. napus* induces distinct genome-wide DNA methylation changes compared with either stress applied individually. These combined stress-specific epigenetic changes are associated with the differential regulation of stress-responsive genes and genomic loci.

Research Objectives

The overall objective of this study was to investigate genome-wide DNA methylation dynamics associated with *V. longisporum* infection and drought stress in *B. napus* using ONT sequencing. Specifically, this thesis aims to:

- 1) characterize genome-wide DNA methylation changes associated with *V. longisporum* infection in *B. napus* to identify differentially methylated loci located at or near defence-related genes involved in host responses to infection; and

2) determine how drought stress modifies genome-wide DNA methylation patterns associated with *V. longisporum* infection in *B. napus* to identify stress-specific differentially methylated loci and their overlap with differentially expressed genes and to evaluate whether these epigenetic changes may contribute to the molecular mechanisms underlying the altered host-pathogen interaction observed under combined stress conditions.

3. GENOME WIDE METHYLOME PROFILING REVEALS CONTEXT-SPECIFIC EPIGENETIC RESPONSES OF *Brassica napus* TO *Verticillium longisporum*

3.1 Author Contributions

Gursimran Kaur performed high-molecular-weight DNA isolation, ONT library preparation, sequencing and methylation data analysis. Gursimran Kaur and Mamta Masand prepare the figures and wrote the initial draft. Dr. Sean Walkowiak helped with ONT data generation and gave critical feedback on the manuscript. Dr. Claudio Stasolla contributed to experimental design and provided guidance for greenhouse experiments and reviewed and edited the manuscript. Dr. Harmeet Singh Chawla secured funding, conceptualized and supervised the project, guided the analyses, and revised and finalized the manuscript. All authors reviewed and approved the final version of the manuscript.

3.2 Abstract

Canola (*Brassica napus* L.) is a commercially important oilseed crop threatened by *Verticillium longisporum*, a soil-borne vascular fungal pathogen. While genetic resistance has been widely studied, the role of epigenetic regulation in plant responses to vascular fungal infection remains poorly understood, particularly in polyploid crop species. This study aimed to characterize genome-wide DNA methylation dynamics in *B. napus* following *V. longisporum* infection using Oxford Nanopore Technologies sequencing. ONT sequencing enabled direct profiling of DNA methylation in infected and mock-inoculated plants. A total of 824, 267, and 19 differentially methylated loci were identified in CG, CHG, and CHH contexts, respectively. DML were distributed across both gene-associated and transposable element regions, with distinct context-specific patterns. Multiple DML overlapped genes associated with plant defence, including those involved in receptor-like kinase signaling, oxidative stress responses, calcium signaling, and transcriptional regulation. In addition, intergenic methylation changes proximal to defence-related genes identify candidate regulatory regions that warrant future functional validation. *V. longisporum* infection was associated with localized and context-dependent changes in DNA methylation in *B. napus*, with most variation occurring in CG and CHG contexts. The association of DML with defence-related genes suggests that DNA methylation may contribute to the regulation of plant responses during vascular fungal infection. These findings provide new insights

into epigenetic responses in a complex polyploid crop and establish a foundation for future studies investigating the role of DNA methylation in plant disease responses.

3.3 Introduction

Brassica napus is one of the most important oilseeds crops globally, valued for its high-quality edible oil and protein-rich meal. It plays a key role in food, feed, and biofuel production and is a key component of temperate agricultural systems (Friedt & Snowden, 2010). However, sustainable *Brassica napus* production is increasingly challenged by soil-borne pathogens that are difficult to manage using conventional agronomic practices, leading to persistent yield losses and reduced crop resilience (Cui et al., 2025).

Among these pathogens, *Verticillium longisporum* has emerged as a major threat to *B. napus* production in Europe and North America (Depotter et al., 2016). This vascular, soil-borne fungal pathogen infects plants through the roots and colonizes the xylem, disrupting water and nutrient transport. *Verticillium longisporum* is a hybrid species complex with distinct lineages that differ in host range and pathogenicity (Inderbitzin et al., 2013). Its hybrid origin is also reflected at the genomic level, and genome assemblies have provided additional insight into the organization and evolution of this pathogen (Depotter et al., 2021). In *B. napus*, infection is associated with symptoms including stunting, stem striping, premature senescence, and significant reductions in biomass and yield. Field studies have reported to cause significant yield reductions under field conditions, with the magnitude of loss depending on environmental conditions and disease severity (Cui et al., 2025; Depotter et al., 2019). The production of long-lived microsclerotia enables the pathogen to persist in soil for extended periods, limiting the effectiveness of chemical control and highlighting the need for alternative strategies to improve disease resilience (Depotter et al., 2016).

Plants exhibit substantial phenotypic plasticity that enables rapid responses to environmental stresses, including pathogen attack. In addition to classical genetic resistance, epigenetic regulation has gained attention as an important mechanism for modulating stress-responsive gene expression in plants (Springer & Schmitz, 2017). Through processes such as DNA methylation, histone modification, and chromatin reconfiguration, transcription can be regulated without any change in the underlying nucleotide sequence. Importantly, stress-associated epigenetic alterations can arise quickly and may sometimes persist after the initial stress event, providing a basis for stress memory

and priming plants for improved responses to future stress exposure (Crisp et al., 2016; Lämke & Bäurle, 2017).

Among the different epigenetic layers, DNA methylation is the most thoroughly investigated epigenetic modifications in plants. It is established at cytosine residues within CG, CHG, and CHH contexts (where H denotes A, T, or C) and contributes to multiple biological functions, including transcriptional regulation, preservation of genome stability, and TE silencing (Law & Jacobsen, 2010; Zhang et al., 2018). Pathogen challenge can provoke dynamic, sequence-context-dependent alterations in DNA methylation, thereby influencing the transcriptional reprogramming of defence-associated genes and the overall host response (Downen et al., 2012; López Sánchez et al., 2016). Previous reports suggested, DNA methylation have been shown to be dynamically regulated at specific genomic loci, with active demethylation contributing to stress-responsive gene regulation in *A. thaliana* (Penterman et al., 2007).

Functional studies, particularly in *Arabidopsis*, further suggest that differential methylation often targets genes involved in hormone signalling, immune perception, and transcriptional regulation (Deleris et al., 2016). For example, methylation changes have been reported at loci encoding components of the salicylic acid pathway, WRKY and bZIP transcription factors, receptor-like kinases, and genes associated with ROS metabolism following pathogen challenge (Downen et al., 2012; López Sánchez et al., 2016; Tirnaz et al., 2022). In general, CG methylation changes are frequently associated with gene-proximal regulatory regions, whereas non-CG methylation (CHG and CHH) is often enriched in TE-associated regions, reflecting complementary roles in gene regulation and genome stability (Zhang et al., 2018). However, the relative contribution of different methylation contexts can vary depending on the host-pathogen system and genomic architecture. Nevertheless, the contribution of DNA methylation to host responses against vascular fungal pathogens like *V. longisporum* is still largely unresolved, especially in polyploid crops such as *Brassica napus*, where genome complexity poses additional challenges. The large genome size, high repeat content, and extensive duplication in *B. napus* present challenges for accurately resolving methylation patterns and associating them with functional genomic elements (Cui et al., 2023).

Although whole-genome bisulfite sequencing provides accurate, base-resolution DNA methylation profiles, its dependence on short-read mapping can reduce coverage and mapping

confidence in repetitive and structurally complex genomic regions (Liu et al., 2019; Zhang et al., 2018). In contrast, the long native reads generated by ONT sequencing can improve read alignment across these regions, enabling methylation states to be assigned more confidently to specific genomic loci that may be difficult to resolve using short-read bisulfite sequencing (Liu et al., 2019; Simpson et al., 2017). Furthermore, it has been directly demonstrated in plant genomes, including the *Brassicaceae* species *B. nigra* where Nanopore-based methylation calls showed high agreement with bisulfite sequencing across all three cytosine contexts while additionally recovering methylation information in repeat-dense regions, including centromeric and pericentromeric regions, that are challenging to resolve using short-read bisulfite sequencing approaches (Ni et al., 2021; Perumal et al., 2020). The long-read nature of ONT sequencing improves mapping accuracy in polyploid genomes and enables simultaneous analysis of sequence and epigenetic modifications at single-molecule resolution (Wang et al., 2021).

In the present study, genome-wide DNA methylation patterns were examined in hypocotyl tissues of *B. napus* cv. Westar following infection with *V. longisporum* isolate UMB_1 (A1/D1 lineage). ONT based direct methylation profiling was applied to characterize pathogen-associated methylation dynamics and identify DML and DMR associated with stress-responsive genomic features. These findings expand current understanding of the epigenetic basis of canola responses to vascular fungal infection and highlight DNA methylation as an important regulatory component of plant stress adaptation. This study represents the first genome-wide analysis of DNA methylation dynamics in *B. napus* during *V. longisporum* infection using long-read sequencing.

3.4 Materials and Methods

3.4.1 Plant system and growth conditions

Seeds of *B. napus* cv. Westar were obtained from Agriculture and Agri-Food Canada (AAFC), Saskatoon Research and Development Centre, 107 Science Place, Saskatoon, Saskatchewan S7N 0X2, Canada (52.13415° N, 106.63491° W). Westar is a canola-quality cultivar developed in Canada and widely used in research and breeding programs. All plant materials used in this study were obtained from cultivated sources, and no wild collections were performed. Therefore, no specific permits or permissions were required. All experimental procedures complied with the

University of Manitoba Faculty of Agricultural and Food Sciences (FAFS) Biosecurity Protocol and applicable institutional guidelines for plant research.

Seeds were sown in 48-cell plastic seedling trays containing Sunshine Mix #4 (Sun Gro Horticulture, Canada), a peat-based soilless medium supplemented with perlite, dolomitic limestone, and gypsum. Seedlings were maintained in a controlled-environment growth chamber under a 16 h light/8 h dark photoperiod at 22 °C/18 °C (day/night). Plants were grown for 14 days until the two-leaf stage prior to inoculation.

3.4.2 Fungal culture

The *V. longisporum* isolate UMB_1 (A1/D1 lineage) was obtained from the laboratory culture collection maintained at the University of Manitoba, Winnipeg, Manitoba, Canada. The isolate was originally recovered from infected canola plants maintained in the University of Manitoba greenhouse and has been previously described and utilized in pathogenicity studies (Kashyap et al., 2026). Fungal cultures were maintained on potato dextrose agar (PDA; Sigma-Aldrich) supplemented with spectinomycin (100 µg mL⁻¹) at 25 °C in darkness for approximately 3 weeks to promote conidial and microsclerotial development. For inoculum preparation, agar plugs taken from the actively growing edge of the colony were transferred into potato dextrose broth containing the same concentration of spectinomycin and incubated in darkness at 23 °C with shaking at 120 rpm for 7-14 days, following previously established procedures (Eynck et al., 2009). The resulting cultures were passed through sterile muslin cloth to remove mycelial fragments. Spore suspensions were either used immediately for inoculation or stored in sterile 20% (v/v) glycerol at -80 °C. Before inoculation, conidial concentration was quantified using a hemocytometer and adjusted to 5 × 10⁵ conidia mL⁻¹ (Kashyap et al., 2026).

3.4.3 Plant inoculation

At 14 days post-sowing, seedlings were carefully uprooted, and the roots were thoroughly rinsed with sterile distilled water to remove attached growth medium. Root ends were then trimmed to a length of approximately 1-2 cm to facilitate infection. The seedlings were subsequently root-dip inoculated in a *V. longisporum* conidial suspension adjusted to 5 × 10⁵ conidia mL⁻¹ for 30 min, consistent with previously described protocols (Eynck et al., 2009). Control seedlings were mock inoculated by immersion in sterile distilled water for an identical period. After treatment, all

seedlings were replanted in fresh soil and maintained under the previously described growth conditions for 5 days prior to sample collection. The experiment was conducted as a completely randomized design with three biological replicates for each treatment. Individual biological replicates consisted of pooled hypocotyl tissues collected from multiple seedlings within the same treatment unit. All treatments were applied and processed simultaneously in a single experimental run under identical environmental conditions.

3.4.4 Sample collection and high-molecular-weight DNA extraction

Hypocotyl tissues were harvested at 5 days post inoculation (dpi) from mock- and *V. longisporum*-inoculated seedlings. The 5-dpi time point was selected because previous observations highlighted that *V. longisporum* had colonized above-ground tissues by this stage, making hypocotyl tissue an active host-pathogen interaction zone (Kashyap et al., 2026). Immediately after excision, tissues were frozen in liquid nitrogen and stored at -80 °C until further processing. High-molecular-weight genomic DNA was extracted using a modified method developed for long-read sequencing applications (Schalamun Miriam & Schalamun Benjamin, 2017). Frozen tissue was finely ground under liquid nitrogen and lysed in a polyvinylpyrrolidone- and sodium dodecyl sulfate-based extraction buffer to reduce oxidation and co-extraction of secondary metabolites. DNA was purified using magnetic bead-based clean-up steps with gentle handling to preserve fragment integrity. DNA quantity and purity were determined using Qubit fluorometric measurements and NanoDrop spectrophotometry, whereas fragment integrity and size distribution were assessed by agarose gel electrophoresis and Agilent TapeStation analysis before library construction. For mock-inoculated samples, genomic DNA was subjected to controlled shearing using Covaris g-TUBEs followed by BluePippin size selection to enrich for fragments >8 kb prior to library preparation. In contrast, DNA from *V. longisporum*-inoculated samples was sequenced without prior shearing or size selection because sufficient high-molecular-weight DNA was recovered directly from these samples. As a result, read-length distributions differed between treatments, but all samples were processed using the same downstream alignment, filtering, and methylation analysis pipeline, and uniform coverage and window-based filtering criteria were applied to ensure comparability between control and inoculated datasets. Purified DNA was stored at -80 °C until library preparation.

3.4.5 Library preparation for Oxford Nanopore Technologies sequencing

Sequencing libraries were constructed from genomic DNA using the ONT ligation sequencing kit with native barcoding (SQK-NBD114.24) in accordance with the manufacturer's protocol (version number: NBA_9168_v114_revT_02Oct2025; <https://nanoporetech.com/document/ligation-sequencing-amplicons-native-barcoding-v14-sqk-nbd114-24?device=PromethION>). The workflow included DNA repair and end-preparation, barcode ligation, and adapter ligation, with magnetic bead-based purification carried out between enzymatic steps. No PCR amplification was included during library preparation in order to retain native DNA methylation patterns.

3.4.6 Oxford Nanopore Technologies sequencing

Oxford Nanopore Technologies sequencing was performed on the P2 Solo platform using R10.4.1 flow cells in accordance with the manufacturer's standard protocol (version number: NBA_9168_v114_revT_02Oct2025; <https://nanoporetech.com/document/ligation-sequencing-amplicons-native-barcoding-v14-sqk-nbd114-24?device=PromethION>). For each treatment, three biological replicates were multiplexed using the native barcoding system, pooled, and loaded onto a single flow cell. Thus, a total of two flow cells were used, with one dedicated to mock-inoculated samples and the other to *V. longisporum*-inoculated samples. Sequencing was continued for up to 72 h or terminated earlier when pore activity showed a substantial decline.

3.4.7 Basecalling, read alignment, and methylation detection

Raw signal data were basecalled using Dorado v7.9.8 with a high-accuracy model and modified-base calling enabled for cytosine methylation detection. Only reads passing the default quality filter were retained for downstream analyses. Basecalled reads for each biological replicate were exported in FASTQ format and aligned to the *B. napus* cv. Westar v0 reference genome downloaded from the BnaOmics portal (Cui et al., 2023) using minimap2 (Li, 2018) with parameters optimized for ONT reads. Resulting alignments were converted to BAM format, sorted, and indexed using samtools (Li et al., 2009). Cytosine methylation information was extracted from aligned BAM files using modkit pileup (Oxford Nanopore Technologies, 2023) generating per-base methylation calls for CG, CHG, and CHH sequence contexts. Although an independent whole-genome bisulfite sequencing benchmark was not performed in this study, several measures support the reliability of the methylation calls. Modified-base calls were filtered using modkit's

empirically derived per-sample confidence threshold, calculated from mapped reads for each sequencing run, yielding confidence thresholds ranging from 0.744 to 0.760 (mean = 0.751)

3.4.8 Differential methylation analysis

Methylation calls were processed separately for the CG, CHG, and CHH sequence contexts for each biological replicate. To facilitate regional methylation analysis and reduce noise associated with individual cytosine measurements, per-base methylation calls generated by modkit were aggregated into non-overlapping 1 kb genomic windows. For each window, methylated read counts and total valid coverage were summed across all cytosines within the corresponding sequence context. Differential methylation analysis was performed independently for each sequence context using the R package DSS (Dispersion Shrinkage for Sequencing data) (Feng & Wu, 2019), which models methylated and total read counts using a beta-binomial framework to account for biological variability among replicates. Replicate-specific window counts were used to construct BSseq objects. Prior to statistical testing, windows were retained only if the minimum total coverage threshold was met in at least two mock-inoculated and two inoculated biological replicates. Minimum coverage thresholds were set to 200, 100, and 50 total reads per 1 kb window for CG, CHG, and CHH contexts, respectively, reflecting differences in cytosine abundance and methylation levels among sequence contexts.

Differentially methylated loci, defined as non-overlapping 1 kb genomic windows exhibiting significant methylation differences between treatments, were identified using the DSS DML test, applying a false discovery rate (FDR) threshold of < 0.05 and a minimum absolute methylation difference (Δ) of ≥ 0.05 . Differentially methylated regions (DMR) were identified independently using the DSS callDMR function on the full DML test output, applying a p-value threshold of 0.001 and the same minimum methylation difference ($\Delta \geq 0.05$); DMR were not constrained to the predefined 1 kb windows. Both thresholds were applied uniformly across the CG, CHG, and CHH contexts. DML were classified as hypermethylated or hypomethylated based on the direction of methylation change in inoculated plants relative to mock-inoculated controls.

To estimate the empirical background signal associated with ONT-based methylation detection, an additional chloroplast-based negative-control analysis was performed. The *B. napus* chloroplast genome (Hu et al., 2011) was used as an internal negative-control reference because chloroplast DNA is generally considered to contain negligible levels of cytosine methylation. Reads from all

samples were aligned to the chloroplast genome using the same alignment workflow employed for the nuclear genome while preserving MM/ML modification tags for downstream methylation calling. Cytosine methylation was identified using modkit with the same parameters applied to the nuclear genome analysis. Chloroplast cytosine positions with a minimum read depth of 10× were considered callable, and methylation-like calls were defined as cytosine positions exhibiting a methylation frequency of $\geq 70\%$. The proportion of methylation-like calls among all callable chloroplast cytosine positions was calculated for each sample as an empirical estimate of the background signal associated with the ONT methylation-calling workflow.

3.4.9 Differentially methylated loci annotation and identification of genes and TEs

Differentially methylated loci were intersected with annotated genomic features using BEDTools (Quinlan & Hall, 2010) to identify loci directly overlapping genes and TEs, based on *B. napus* genome annotations (Yang et al., 2023). To capture potential regulatory regions, DML located within ± 2 kb of annotated gene coordinates but not overlapping gene bodies were classified as intergenic and assigned to the nearest gene based on genomic proximity. DML were therefore categorized as either gene-associated (overlapping gene bodies), TE-associated elements, or intergenic (located within ± 2 kb of genes without direct overlap). Functional annotations of associated genes were retrieved for downstream analysis (Yang et al., 2023).

3.5 Results

3.5.1 Sequencing depth and read quality

High-quality long-read sequencing data were generated for all six samples, comprising three mock-inoculated and three *V. longisporum*-inoculated biological replicates. For the mock-inoculated samples, sequencing produced 30.3 Gbp, 22.3 Gbp, and 25.5 Gbp of passed data for replicates 1, 2, and 3, corresponding to approximate genome coverages of 26.3×, 19.3×, and 22.17×, respectively, based on the *B. napus* cv. Westar reference genome. For the *V. longisporum*-inoculated samples, sequencing yielded 28.9 Gbp, 32.1 Gbp, and 32.9 Gbp of passed data for replicates 1, 2, and 3, corresponding to genome coverages of approximately 25.13×, 27.91×, and 28.66×, respectively. Read length distributions were consistent across mock samples, with an average read N50 of ~ 10.5 kb, whereas inoculated samples exhibited a lower average read N50 of ~ 6.0 kb (Table S3.1). Despite this difference, sequencing depth was comparable across treatments

and sufficient to support downstream methylation analyses. To estimate the empirical background signal associated with ONT-based methylation detection, reads from all six samples were aligned to the *B. napus* chloroplast genome, used as an internal negative control, and analysed using the same methylation-calling workflow applied to the nuclear genome. Chloroplast primary mapping rates were consistent across biological replicates, ranging from 3.99-4.10% in mock-inoculated samples and 5.42-5.48% in *V. longisporum*-inoculated samples. Across all six samples, a combined total of 333,074 chloroplast cytosine positions met the minimum coverage threshold ($\geq 10\times$), of which only 15 exhibited methylation frequencies $\geq 70\%$, corresponding to a pooled empirical background rate of 0.0045%. This background rate was consistently low across both treatment groups (0.0036-0.0072% in mock-inoculated samples; 0.0036% in all three *V. longisporum*-inoculated replicates), indicating that the ONT methylation-calling workflow produces minimal false-positive signal independent of infection status.

3.5.2 Global DNA methylation changes across cytosine contexts

To assess *V. longisporum*-associated epigenomic changes, DNA methylation was analyzed in CG, CHG, and CHH sequence contexts across the genome. Differential methylation analysis was performed using the DSS framework on 1 kb genomic windows generated from ONT-derived methylation calls. After coverage filtering, 798,272 of 960,735 CG windows (83.09%), 912,599 of 961,824 CHG windows (94.88%), and 942,472 of 962,870 CHH windows (97.88%) were retained for downstream analysis. Using a significance threshold of $FDR < 0.05$, DSS analysis identified 824 DML and 31 DMR in the CG context, 267 DML and 11 DMR in the CHG context, and 19 DML and 1 DMR in the CHH context between *V. longisporum*-inoculated and mock-inoculated plants (Table S3.2.). Genome-wide distribution of DML associated with *V. longisporum* infection in *B. napus* is illustrated in Figure 3.1.

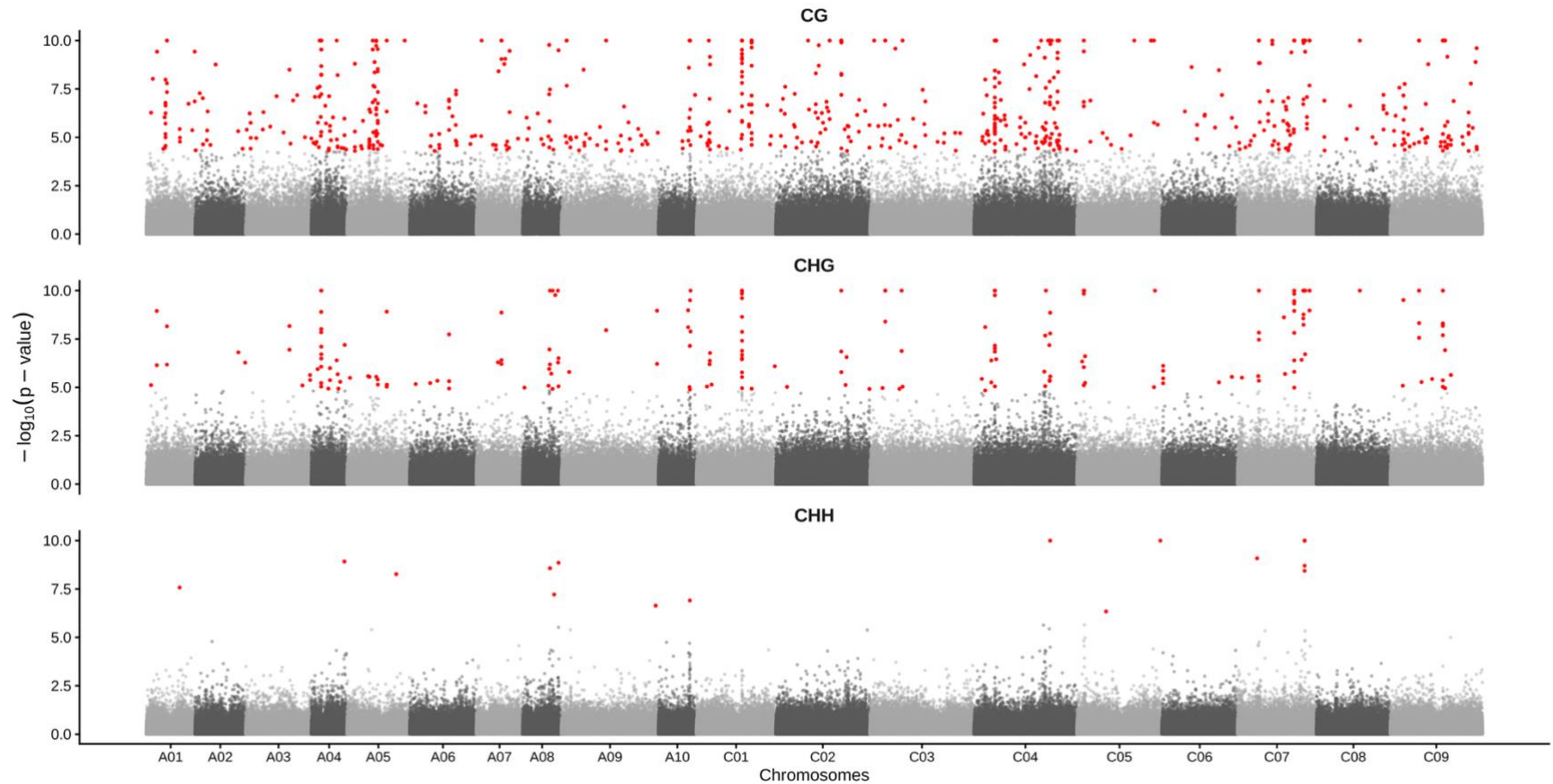


Figure 3.1 Genome-wide distribution of differentially methylated loci associated with *Verticillium longisporum* infection in *Brassica napus* L. Differentially methylated loci were identified by comparing mock-inoculated control plants and *V. longisporum*-infected plants and are shown separately for the CG, CHG, and CHH methylation contexts. Genomic positions of loci across the A and C subgenomes chromosomes are shown on the x-axis, while the statistical significance of differential methylation is shown as $-\log_{10}(p\text{-value})$ on the y-axis. Significant DML are highlighted in red, whereas background loci are shown in grey.

All identified DMR across CG, CHG, and CHH contexts exhibited positive methylation differences, suggesting that these regions were hypomethylated in inoculated plants relative to mock-inoculated controls. These results indicate that pathogen infection was associated with measurable methylation changes across all cytosine contexts, with the CG context exhibiting the largest number of alterations.

3.5.3 Directionality and context specificity of differential methylation

The direction and magnitude of methylation changes varied across cytosine contexts. In the CG context, *V. longisporum*-inoculated plants exhibited a greater proportion of hypomethylated loci (516; 62.6%) than hypermethylated loci (308; 37.4%) relative to mock-inoculated controls. A similar trend was observed in the CHG context, where 197 loci (73.8%) were hypomethylated and 70 loci (26.2%) were hypermethylated. In contrast, only a limited number of CHH-associated DML were identified, with a nearly balanced distribution of 10 hypomethylated (52.6%) and 9 hypermethylated (47.4%) loci. Volcano plots further illustrated this context-dependent shift in methylation direction, highlighting the predominance of hypomethylation in CG and CHG contexts and the comparatively weak directional bias observed among the limited set of CHH-associated DML (Supplemental Figure S3.1). Overall, these results indicate that *V. longisporum* infection is associated primarily with CG and CHG-context hypomethylation, whereas the limited number of CHH-associated DML showed no strong directional bias.

3.5.4 Genomic distribution of differentially methylated loci

To examine the genomic distribution of methylation changes, DML were intersected with annotated gene and TE regions in the *B. napus* cv. Westar genome to identify loci directly overlapping genomic features. Circos-based genome-wide visualization showed that DML were distributed across multiple chromosomes and occurred in both gene-associated and TE-associated regions, with clear context-dependent differences in localization (Figure 3.2).

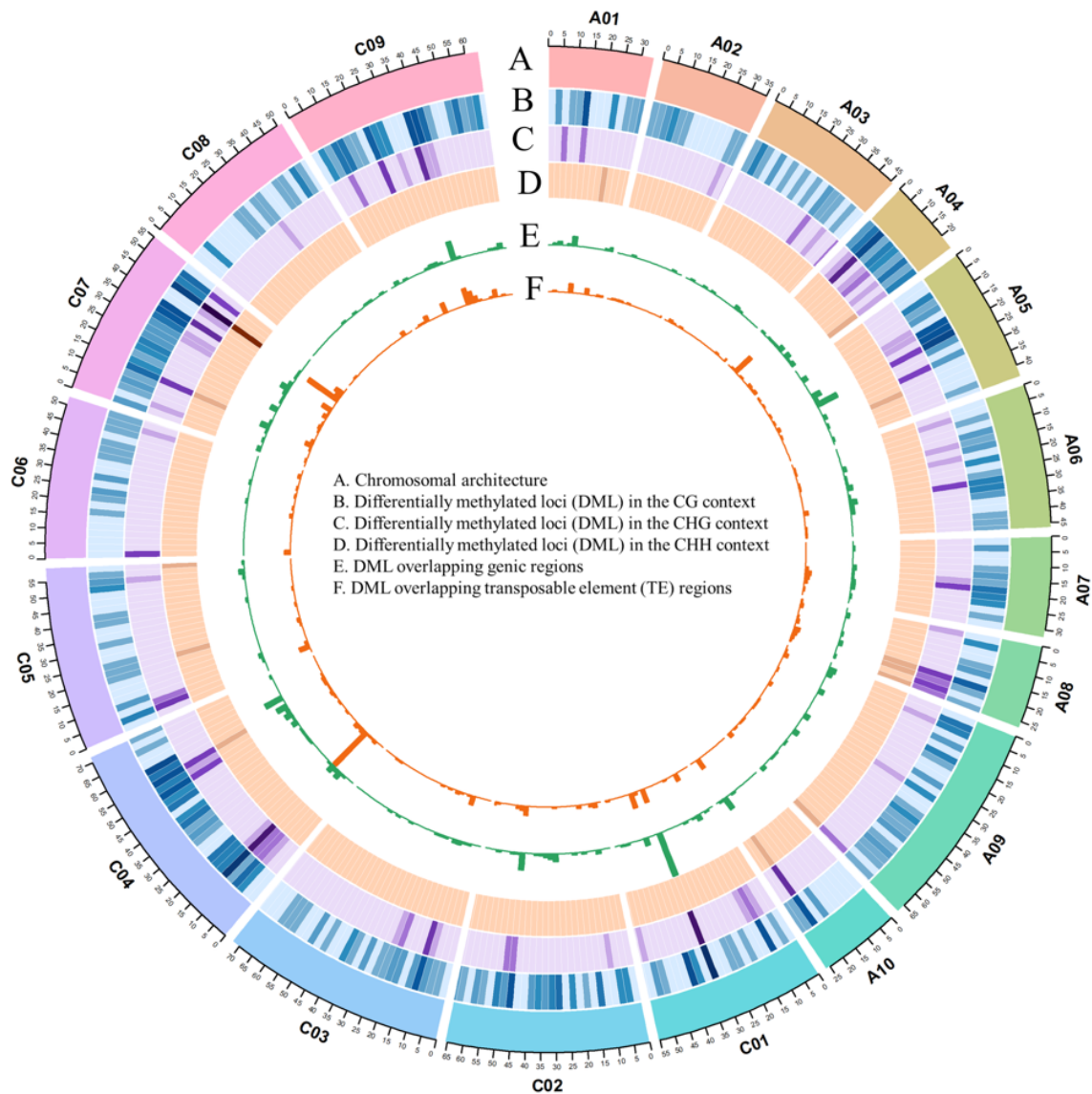


Figure 3.2 Circos representation of genome-wide DML associated with *Verticillium longisporum* infection in *Brassica napus* L.

Differentially methylated loci were identified by comparing control and *Verticillium longisporum* infected plants. The outermost track A. depicts chromosome organization with genomic coordinates in megabases (Mb). Tracks B-D show the genomic distribution of DML in the CG, CHG, and CHH sequence contexts, respectively. Tracks E and F indicate the density of DML overlapping genic regions and TE regions, respectively.

In the CG context, 338 DML (41.01%) overlapped transposable elements and 308 (37.4%) overlapped gene regions, suggesting that CG methylation changes occur across both repetitive and gene-associated regions. Of the gene-overlapping CG-context DML, the majority (249) were located within exonic sequence only, while 13 were located within gene bodies without exon overlap, consistent with intronic positioning. A subset of DML was located within untranslated regions, with 19 DML overlapping 5' UTRs and 31 overlapping 3' UTRs. In the CHG context, 152 DML (56.93%) overlapped TEs and 88 (32.95%) overlapped genes, showing a higher proportion of CHG methylation changes within TE regions. Of the gene-overlapping CHG-context DML, the majority (71) were located within exonic sequence only, while 11 were located within gene bodies without exon overlap, consistent with intronic positioning. A small number of DML were located within untranslated regions, with 2 DML overlapping 5' UTRs and 4 overlapping 3' UTRs. In contrast, only a limited number of CHH-associated DML were identified, though these showed a higher proportion of overlap with TEs, with 13 DML (68.42%) in TE regions and 5 (26.31%) in gene regions. Of the limited number of gene-overlapping CHH-associated DML, four were located within exonic sequence only, and one additionally overlapped a 5' UTR; no CHH-associated DML overlapped a 3' UTR. Metaplot analysis further supported these distribution patterns by showing the average methylation profiles of DML-associated regions across genes and flanking sequences, highlighting context-dependent differences in methylation behavior around annotated genomic features (Figure 3.3A, B). Because *B. napus* is an allotetraploid comprising the A_n and C_n subgenomes, the frequency of significant DML was compared between the two subgenomes after accounting for differences in the number of tested windows. In the CG context, 257 of 314,208 A_n -subgenome windows (0.082%) and 509 of 463,723 C_n -subgenome windows (0.110%) were identified as significant DML, corresponding to a 1.34-fold higher frequency of DML in the C_n subgenome relative to the A_n subgenome ($\chi^2 = 14.88$, $P = 0.00011$). A similar pattern was observed in the CHG context, where 78 of 361,227 A_n -subgenome windows (0.022%) and 153 of 526,190 C_n -subgenome windows (0.029%) were identified as significant DML, representing a 1.35-fold higher frequency in the C_n subgenome ($\chi^2 = 4.61$, $P = 0.032$).

In contrast, no significant difference in DML frequency was detected between subgenomes in the CHH context (8 of 371,653 A_n -subgenome windows, 0.0022%; 9 of 542,322 C_n -subgenome windows, 0.0017%; $\chi^2 = 0.29$, $P = 0.591$). However, only 17 chromosome-assigned CHH DML were identified, limiting the statistical power to detect subgenome-specific differences in this

context. A complete summary of DML and tested windows assigned to each subgenome, together with loci located on unplaced scaffolds. A subset of DML did not overlap annotated gene or TE regions and are therefore likely located within intergenic or unannotated genomic regions. In addition, DML located within ± 2 kb of gene coordinates was identified as intergenic and considered as putative regulatory loci associated with nearby genes. Overall, methylation changes in response to *V. longisporum* infection were distributed across both gene-associated and transposable element regions, with additional loci located in proximal intergenic regions, indicating that methylation changes are not restricted to annotated functional elements.

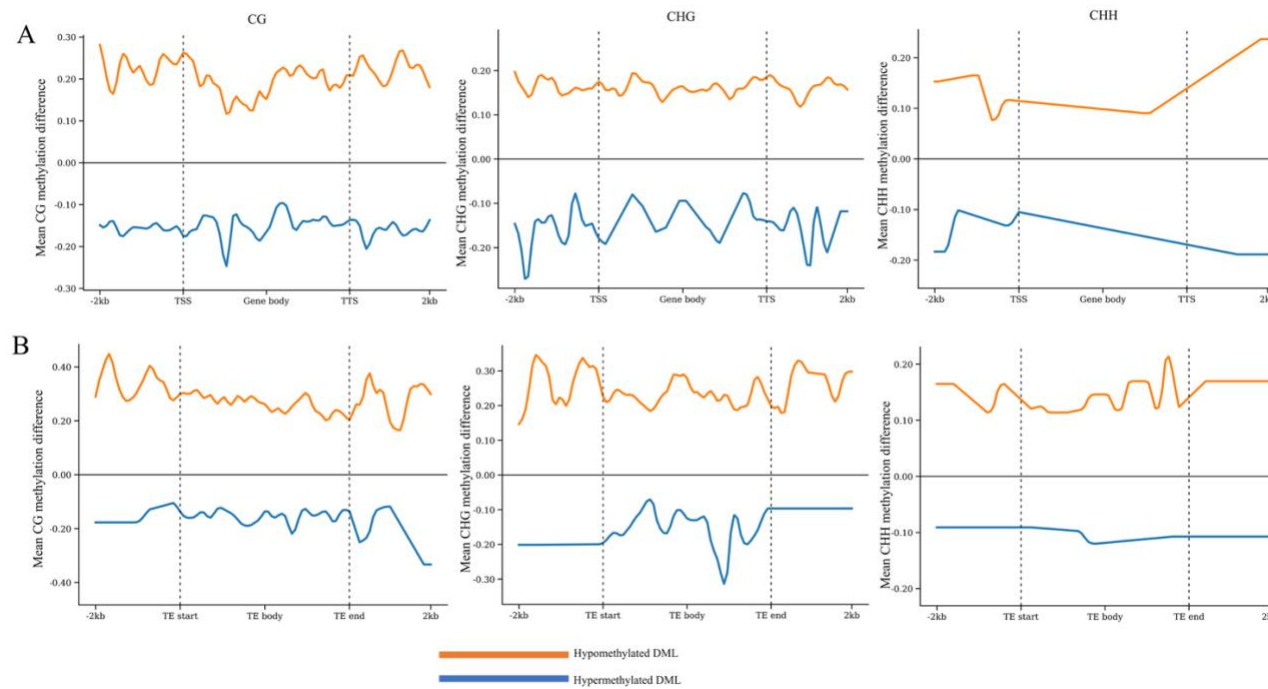


Figure 3.3 Metaplot analysis of differential DNA methylation patterns across genes and transposable elements (TEs)

Metaplots showing the mean methylation difference of hypomethylated and hypermethylated DML across genic and transposable element (TEs) regions, identified by comparing mock inoculated and *Verticillium longisporum* infected plants. A. Average methylation differences across gene-associated regions, including the 2 kb upstream region, transcription start site (TSS), gene body, transcription termination site (TTS), and 2 kb downstream region, shown separately for CG, CHG, and CHH contexts. B. Average methylation differences across TE-associated regions, including the 2 kb upstream flank, TE start, TE body, TE end, and 2 kb downstream flank, for CG, CHG, and CHH contexts. Orange lines represent hypomethylated DML, whereas blue lines represent hypermethylated DML. Dashed vertical lines indicate feature boundaries.

3.5.5 Defence-related differentially methylated genes

Annotation of genes overlapping DML identified multiple candidate loci with predicted or previously described roles in defence- and stress-associated processes. In the CG context, both hyper- and hypomethylated DML overlapped genes annotated for pathogen perception, signaling, and stress-response functions. Representative hypermethylated genes included *BnaC02G0309300WE* (*WAKL22*), *BnaC02G0454400WE* (*LRR*), and *BnaC01G0420300WE* (*CALS9*), while hypomethylated genes included *BnaC02G0289800WE* (*receptor-like kinase*) and *BnaA06G0279100WE* (*CMTA6*). In the CHG context, DML-associated genes included loci annotated for receptor signaling, calcium-related regulation, and cellular transport, including *BnaA02G0335800WE* (*NORK-like receptor kinase*) and *BnaC07G0070300WE* [*DTX1* (*MATE transporter*)]. These results indicate that DML occur within or near genes associated with multiple defence- and stress-related processes, including pathogen recognition, intracellular signaling, and cellular stress responses. However, these genes should be considered candidate methylation-associated loci, and additional transcriptomic or functional validation will be required to determine whether the observed methylation changes are associated with altered gene expression. Representative examples of DML overlapping defence-associated genes were shown in Figure 3.4 and Supplemental Figure S3.2.

3.5.6 Context-specific association of intergenic DML with defence-related genes

To investigate potential regulatory effects of DNA methylation, DML located in intergenic regions within ± 2 kb of annotated genes was analyzed. Distinct patterns were observed across CG, CHG, and CHH contexts. In the CG context, a subset of DML was associated with genes involved in immune regulation, including *Enhanced Disease Susceptibility 1* (*EDS1*), leucine-rich repeat (LRR) proteins, and TIR domain-containing proteins, which are known to function in pathogen recognition and ETI. In the CHG context, intergenic DML included genes associated with defence-related processes, such as cytochrome P450 enzymes, protein kinases, U-box domain-containing proteins, and Nramp transporters. Only a limited number of CHH-associated DML were identified near genes, including glycosyl hydrolase family 17 proteins and protein kinases, both of which have been implicated in stress responses. These observations identify candidate genes located near context-specific methylation changes across different sequence contexts.

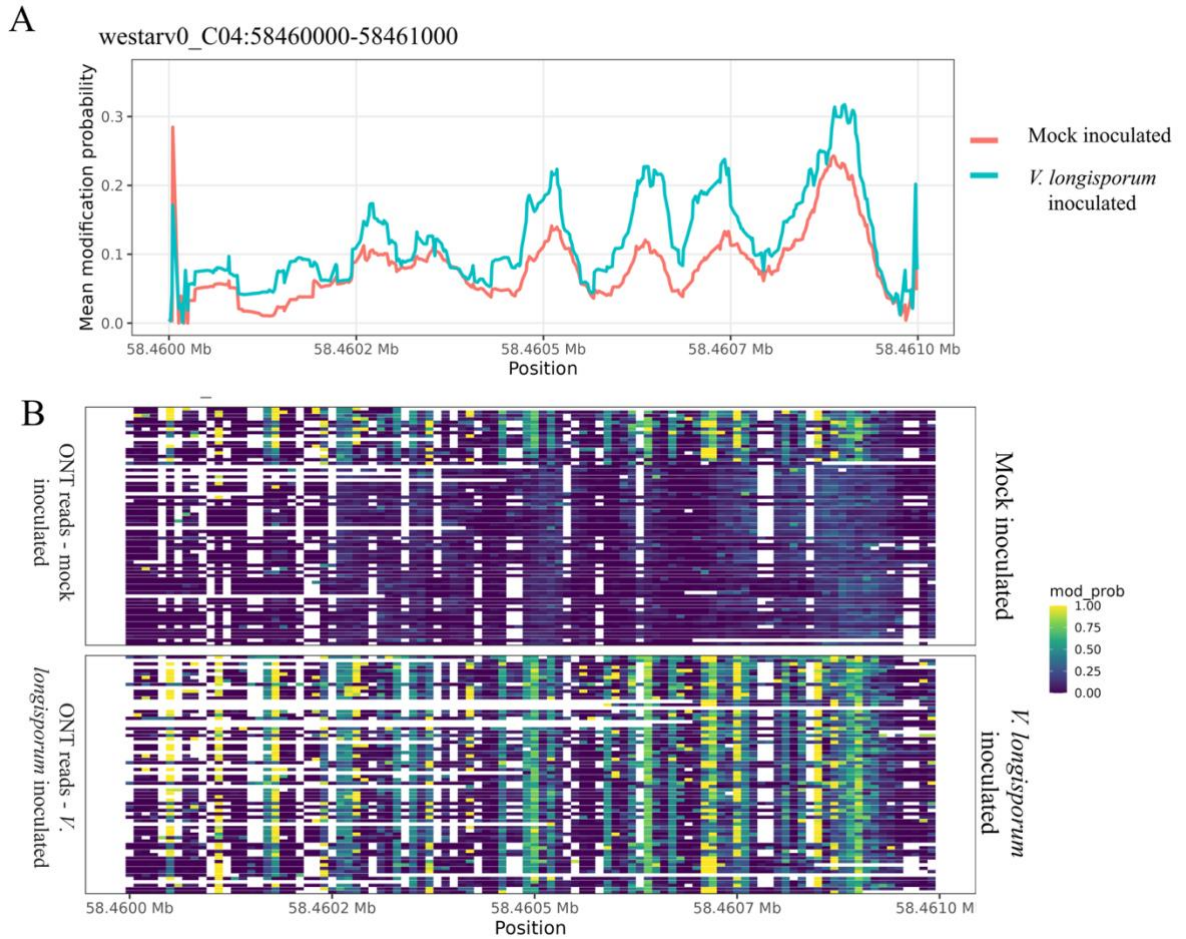


Figure 3.4 Representative DML visualized using NanoMethViz.

A CG-context DML at chromosome C04, positions 58,460,000-58,461,000 bp identified between mock-inoculated and *Verticillium longisporum*-inoculated *Brassica napus* L. samples is shown.

A. Mean methylation modification probability across the locus in the two samples. B. Heatmaps of read-level methylation modification probability across the same region, where each row represents an individual read and colour intensity indicates modification probability. This locus shows clear methylation differences associated with *Verticillium longisporum* inoculation.

3.6 Discussion

3.6.1 Long-read sequencing enables high-resolution epigenetic profiling in a complex crop genome

Oxford Nanopore Technologies sequencing was used in this study to enable direct detection of DNA methylation from native DNA molecules without bisulfite conversion. Unlike bisulfite-based approaches, which rely on chemical treatment and amplification steps that can introduce DNA degradation and sequence bias, ONT infers cytosine methylation directly from raw electrical signals, allowing simultaneous detection of CG, CHG, and CHH methylation contexts within individual reads (Liu et al., 2019; Sigurpalsdottir et al., 2024). Although direct benchmarking of the ONT methylation calls against an orthogonal reference method, such as whole-genome bisulfite sequencing, was beyond the scope of this study, multiple quality-control measures support the robustness of the methylation analyses presented here. The low empirical background signal estimated from the chloroplast negative control indicates that spurious methylation calls were minimal under the analysis parameters used, while the application of modkit's empirically derived per-sample confidence thresholds ensured consistent filtering stringency across biological replicates.

The long-read capability of ONT provides a particular advantage for methylome analysis in *B. napus*, a recent allopolyploid genome formed from hybridization of the A_n and C_n subgenomes, which retains extensive duplication and homoeologous exchange (Chalhoub et al., 2014). Short-read bisulfite sequencing can leave cytosines in repeat-dense and centromeric regions uncalled or under-represented, whereas ONT-based methods have been shown to profile substantially more cytosines in these regions across plant genomes (Chen et al., 2025; Ni et al., 2021). Long-read assemblies similarly capture a greater proportion of the repetitive genome fraction than short-read assemblies in related *Brassica* species (Perumal et al., 2020), improving the genomic context available for methylation profiling in polyploid systems.

Although ONT sequencing currently has lower per-base accuracy than short-read platforms, its ability to simultaneously capture sequence and methylation information from long, native DNA molecules make it well suited for context-specific methylome analysis in complex plant genomes. The methylation patterns observed in this study are consistent with established features of plant

methyomes and may also reflect tissue-specific responses, as hypocotyl tissue represents a key site of vascular colonization during *V. longisporum* infection (Kashyap et al., 2026).

3.6.2 Pathogen-induced methylation changes are localized and context-dependent

Genome-wide analysis of DNA methylation revealed that *V. longisporum* infection is associated with measurable but selective epigenetic variation across the *B. napus* genome. Differential methylation was detected in all three cytosine contexts (CG, CHG, and CHH), indicating that pathogen challenge is associated with multiple layers of the plant methylome. However, the extent of these changes differed among contexts. The majority of DML were identified in the CG context, followed by CHG, while comparatively few changes were detected in the CHH context. This distribution should be interpreted in the context of baseline methylation patterns, as CG methylation is generally more prevalent and stable, whereas CHH methylation is more dynamic and typically occurs at lower levels in plant genomes (Law & Jacobsen, 2010; Zhang et al., 2018). The lower number of CHH-associated changes may therefore reflect the generally lower abundance and dynamic nature of CHH methylation, rather than a reduced response to pathogen infection (Matzke & Mosher, 2014; Stroud et al., 2014). Consistent with previous studies, methylation changes were restricted to a subset of genomic regions rather than occurring across the entire genome (Downen et al., 2012; Secco et al., 2015). Both CG and CHG contexts showed a higher proportion of hypomethylated loci relative to hypermethylated loci, whereas the limited number of CHH-associated DML did not exhibit a strong directional bias.

Together, these observations indicate that DNA methylation responses to *V. longisporum* infection are selective and context-dependent, with the observed differences among contexts reflecting both biological responses and intrinsic properties of cytosine methylation in plant genomes.

3.6.3 Differential methylation is more frequent in the C_n sub genome

Brassica napus is an allotetraploid composed of the A_n and C_n subgenomes, the frequency of pathogen-associated methylation changes was investigated in each sub genome. After normalizing for the number of coverage-filtered windows tested, both CG and CHG DML occurred at significantly higher frequencies in the C_n sub genome than in the A_n sub genome (1.34-fold, $\chi^2 = 14.88$, $P = 0.00011$; and 1.35-fold, $\chi^2 = 4.61$, $P = 0.032$, respectively). Although statistically

significant, the magnitude of this bias was modest, indicating that differential methylation occurred in both subgenomes but was slightly more frequent in the C_n sub genome. No comparable difference was detected in the CHH context ($\chi^2 = 0.29$, $P = 0.591$), although the limited number of chromosome-assigned CHH DML restricted the power to detect sub genome-specific differences. Previous work has displayed that the C_n sub genome exhibits higher baseline levels of CG and CHG methylation than the A_n sub genome in multiple *B. napus* tissues, a pattern that has been attributed in part to the greater abundance of repetitive sequences and transposable elements within the C_n genome (Ziegler et al., 2023). The similar direction and context-specificity of the sub genome bias observed in the present study therefore suggest that the higher frequency of CG- and CHG-associated DML in the C_n sub genome may reflect, at least in part, pre-existing differences in sub genome genomic architecture rather than methylation changes induced specifically by *V. longisporum* infection.

Consequently, although pathogen infection was associated with differential methylation in both subgenomes, the observed C_n-sub genome bias should be interpreted within the context of these inherent genomic differences. Distinguishing baseline sub genome asymmetry from infection-induced methylation responses will require future studies that directly compare the magnitude and distribution of methylation changes between mock-inoculated and infected plants within each sub genome.

3.6.4 Context-specific roles of DNA methylation in plant Defence responses

CG methylation changes were observed across both gene-associated and TE regions, consistent with its widespread presence in plant genomes and its association with transcriptional and chromatin dynamics (Zhang et al., 2018). CHG methylation exhibited a moderate level of variation and showed a notable association with gene regions. Although CHG methylation is typically linked to heterochromatic regions and TEs, it is maintained through interactions with histone H3K9 methylation, forming a reinforcing epigenetic loop that contributes to chromatin stability (Law & Jacobsen, 2010; Stroud et al., 2014). The presence of CHG methylation changes within gene-associated regions in this study suggests that CHG methylation may also be associated with gene-proximal regions in certain genomic contexts.

In contrast, only a limited number of CHH-associated DML were identified. CHH methylation is primarily established through the RNA-directed DNA methylation (RdDM) pathway and is

generally dynamic and locus-specific (Matzke & Mosher, 2014). The restricted CHH response observed here may reflect the inherently lower abundance and dynamic nature of CHH methylation, rather than a reduced response to pathogen infection. To minimize spurious calls in this lower-abundance context, conservative, context-specific thresholds were applied throughout the analysis, including a minimum total coverage of 50 reads per window, met in at least two biological replicates per group, together with an $FDR < 0.05$ and a minimum methylation difference of 0.05. Nevertheless, CHH methylation occurs at substantially lower baseline levels than CG and CHG methylation and is more sensitive to sequencing depth and signal variability in ONT-based methylation detection. Consequently, the CHH-context differences identified in this study should be regarded as exploratory observations and interpreted with greater caution than those identified in the CG and CHG contexts. Additional studies using greater sequencing depth and independent validation approaches will be required to determine whether these CHH methylation differences represent robust biological changes associated with pathogen infection. Together, these findings indicate that CG and CHG methylation account for the majority of detectable variation in this system, while CHH-associated variation appears more limited and context-dependent.

3.6.5 Genomic distribution of methylation changes across genes and transposable elements

Building on these context-specific patterns, the genomic distribution of DML revealed that methylation changes associated with *V. longisporum* infection were distributed across both gene-associated and TE regions, with clear context-dependent patterns. In the CG context, a larger proportion of DML overlapped TEs compared with gene regions, indicating that CG methylation changes are not restricted to genic compartments but are also present within repetitive regions of the *B. napus* genome. This distribution is consistent with the widespread presence of CG methylation across both genes and TEs in plant genomes (Fang et al., 2022).

In contrast, CHG methylation changes showed a slightly higher proportion of overlap with gene regions than with TEs. This pattern is notable given that CHG methylation is typically enriched in heterochromatic regions and TEs. The observed distribution suggests that CHG methylation may also extend to gene-proximal regions in this system, rather than being confined to structural genome silencing. Such gene-associated CHG methylation has been reported in complex plant

genomes (Bewick & Schmitz, 2017) and may reflect increased epigenetic flexibility in polyploid species such as *B. napus* (Zhang et al., 2018)

CHH methylation changes, although limited in number, showed a higher proportion of overlap with TEs relative to gene regions. While this small set of loci should be interpreted with caution, the pattern is broadly consistent with the established role of CHH methylation in transposable element silencing, primarily mediated through RNA-directed DNA methylation (RdDM) (Law & Jacobsen, 2010; Zhang et al., 2018). Overall, these findings indicate that methylation changes in response to pathogen infection are distributed across both functional (genic) and structural (TE) genomic compartments in a context-dependent manner.

3.6.6 Epigenetic association with defence-related genes and regulatory regions

Consistent with these distribution patterns, DML were identified within gene bodies and in nearby regulatory regions, including intergenic regions within ± 2 kb of gene coordinates. A subset of these loci was associated with genes with annotations related to plant immunity and stress responses. These genes represent multiple components of plant Defence pathways, including pathogen perception, signal transduction, and downstream stress responses. For example, receptor-like kinases such as *WAKL22* and cysteine-rich receptor-like kinases such as *CRK36* are known components of plant immune signalling networks (Boller & Felix, 2009). Additional genes identified in this study were associated with processes such as ROS metabolism (Torres et al., 2006), calcium signalling (Dodds & Rathjen, 2010), and cell wall modification, which are commonly implicated in plant responses to pathogen challenge.

Notably, several defence-associated genes identified in this study exhibited hypermethylation. In certain genomic contexts, increased DNA methylation has been associated with altered transcriptional activity, particularly when occurring in gene-proximal or regulatory regions (Downen et al., 2012). In the context of a susceptible interaction, such patterns could reflect misregulated host Defence responses or potential pathogen-mediated modulation of host regulatory pathways. DNA methylation has been associated with transcriptional regulation under biotic stress conditions (Downen et al., 2012). However, given that this represents a susceptible interaction, these associations may reflect a combination of host Defence responses and pathogen-influenced reprogramming of host cellular processes.

3.6.7 Context-specific regulatory potential of intergenic DML near defence-related genes

In addition to gene-associated methylation changes, a subset of DML was identified in intergenic regions located within ± 2 kb of gene coordinates, corresponding to putative regulatory regions such as promoters and other cis-regulatory elements. DNA methylation in these regions has been associated with transcriptional regulation in plants, particularly under stress conditions where methylation changes may influence gene expression without altering coding sequences (Khan et al., 2023; Zhang et al., 2018). When examined in a context-specific manner, a subset of intergenic DML were located near genes associated with diverse functional categories. In the CG context, these loci were found near genes involved in pathogen perception and immune signalling. In contrast, CHG-associated intergenic DML were located near genes linked to downstream defence-related processes such as signalling and metabolism, while CHH-associated intergenic DML were observed near genes implicated in more general stress-responsive functions. These patterns are consistent with previous observations that different methylation contexts are associated with distinct genomic and functional roles in plants (Fang et al., 2022; Liu et al., 2022; Zhang et al., 2018).

Although these associations are based on genomic proximity rather than direct functional validation, the presence of intergenic methylation changes near genes with stress- and defence-related functions may represent candidate loci where methylation may contribute to context-dependent regulatory patterns during *V. longisporum* infection (Zhang et al., 2018).

3.6.8 Potential relevance to crop resilience and future breeding applications

The identification of defence-associated loci exhibiting differential methylation has important implications for crop improvement. In polyploid species such as *B. napus*, where genetic redundancy can buffer the effects of sequence variation, epigenetic regulation may provide an additional layer of variability contributing to stress adaptation. The loci identified in this study represent candidates for further investigation as potential markers associated with disease response.

Moreover, long-read sequencing enables the generation of high-resolution methylation maps that can be integrated with genomic and phenotypic datasets. Such integrative approaches could eventually inform the development of epigenetic markers or breeding strategies aimed at

improving disease resilience in canola and other crop species, contingent on future work establishing the heritability and transgenerational stability of pathogen-induced methylation changes, which remain undetermined here. These findings provide a foundation for future studies investigating whether epigenetic information can be incorporated into breeding frameworks. Such applications will require demonstrating that the identified methylation changes are functionally relevant, stable, and heritable across generations, none of which were assessed in the present study.

3.6.9 Future directions

A limitation of the present study is the use of whole hypocotyl tissue for methylation profiling. As *V. longisporum* colonizes the vascular system, infection-associated methylation changes are likely concentrated within vascular and pathogen-colonized cell types. Consequently, the methylation patterns reported here represent an average across multiple cell populations and may not fully capture localized, cell-type-specific epigenetic responses to colonization. Future studies employing tissue microdissection or single-cell/single-nucleus methylome approaches may provide greater resolution of DNA methylation dynamics during vascular fungal infection.

A further direction for future work involves increasing sequencing depth to better resolve CHH-context methylation differences. Although conservative context-specific coverage thresholds and statistical criteria were applied throughout this study to reduce spurious calls in this lower-abundance context, CHH methylation remains inherently more sensitive to sequencing depth and signal variability than CG and CHG methylation. Increasing sequencing depth, together with independent validation using complementary methylation profiling approaches where feasible, would improve confidence in the detection of biologically meaningful CHH methylation changes and help distinguish genuine pathogen-associated variation from technical noise.

In addition to improving spatial resolution, future research should focus on establishing the functional significance of the methylation changes identified in this study. While candidate loci associated with defence responses were identified, the biological consequences of these methylation changes remain to be experimentally validated. Integrating methylation profiling with transcriptomic, genetic, and functional analyses will be essential for determining the relationships between epigenetic variation and gene expression during *V. longisporum* infection. Such integrative approaches may ultimately facilitate the development of epigenetic markers and

improve understanding of how DNA methylation contributes to disease resilience in canola and other crop species.

3.7 Conclusion

In summary, *V. longisporum* infection is associated with localized and context-dependent DNA methylation changes in *B. napus*, with CG and CHG methylation accounting for the majority of observed variation. These changes occur within gene bodies and in nearby regulatory regions associated with genes involved in plant immunity and stress responses, reflecting both host responses and pathogen-associated processes in a susceptible interaction. The identification of these loci highlights candidate regions where DNA methylation may contribute to regulatory processes during plant-pathogen interactions. Long-read sequencing provided insight into context-specific epigenetic variation in a complex crop genome and may inform future efforts to integrate epigenetic information into plant disease research, pending validation of the stability and heritability of these methylation changes across generations.

3.8 From Pathogen-Induced Methylation to Combined Stress Responses

The results described in Objective 1 highlighted that *V. longisporum* infection is associated with targeted DNA methylation changes in *B. napus*, including methylation variation in genes linked to defence, signalling, and stress responses. These findings are consistent with growing evidence that DNA methylation is dynamically regulated during plant-pathogen interactions (Dowen et al., 2012; Xiao et al., 2021).

Although Objective 1 provided insights into methylation responses associated with pathogen infection, crops in agricultural systems are rarely exposed to a single stress factor in isolation, and disease outcomes are therefore shaped by the prevailing environmental conditions under which infection occurs. Consequently, pathogen-induced methylation changes alone are insufficient to explain plant responses under field conditions. This is illustrated by recent work from our laboratory showing that drought stress restricts the acropetal movement of *V. longisporum* from roots into aerial tissues of *B. napus* and reduces disease severity, demonstrating that water availability can fundamentally reshape the host-pathogen interaction (Kashyap et al., 2026). The molecular mechanisms underlying this drought-modified response, however, remain unclear.

To address this knowledge gap, Objective 2 extends the investigation from pathogen-induced methylation changes to a combined drought- *V. longisporum* system. By integrating transcriptomic and methylome analyses, this objective aims to identify molecular processes associated with drought-mediated changes in pathogen colonization and host responses. Linking epigenetic variation with transcriptional reprogramming provides an opportunity to determine whether stress-associated methylation changes are associated with altered defence responses under combined stress conditions and to improve our understanding of the regulatory mechanisms underlying drought-induced suppression of disease development in *B. napus*.

4. DROUGHT-MEDIATED MITIGATION OF *Verticillium longisporum* COLONIZATION IN *Brassica napus* IS ASSOCIATED WITH TRANSCRIPTOME AND DNA METHYLATION REPROGRAMMING

4.1 Author Contribution

Shruti Kashyap and Gursimran Kaur conducted the experiments, interpreted the data, and drafted the manuscript. Dr. Sean Walkowiak assisted with the infection assays and contributed to the epigenetic and transcriptomic experiments. Dr. Mamta Masand and Natalie Cale supported the analysis of transcriptomic and epigenetic data. Dr. Harmeet Singh Chawla and Dr. Claudio Stasolla conceptualized the study, contributed to data interpretation, and provided critical input during manuscript preparation.

4.2 Abstract

Climate is becoming more variable, and the frequency and magnitude of drought and pathogen epidemics are changing. This creates a need to understand how limited water availability influences plant responses to biotic challenges. In *Brassica napus*, drought decreases the disease progression and fungal colonization of the vascular pathogen *Verticillium longisporum*, yet the underlying regulatory mechanisms remain unclear. Here, we used an integrative approach combining transcriptome profiling, co-expression network analysis, and DNA methylation analysis to investigate host responses in hypocotyl tissues of water-stressed plants at early and later stages of infection. Relative to infected hypocotyls of well-watered plants, imposition of drought evokes extensive transcriptome rewiring centered around three distinct events: the activation of detoxification/defence pathways, including glucosinolate and glutathione metabolism and redox-related processes, induction of cell wall components contributing to formation of apoplastic barriers, and hormonal responses linked to ethylene signalling. Many of these changes were temporally regulated. Co-expression network analysis in infected water-stressed tissue identified tightly connected regulatory modules and hub genes, including receptor-like kinases and transcription factors, indicating coordinated control of stress-responsive pathways. DNA methylation profiling following combined imposition of drought and fungus infection revealed predominantly localized and context-dependent changes, with DML associated with genes annotated to diverse stress-related functions, including defence signalling, hormone-related

pathways, and cell wall processes. Integration of methylome and transcriptome data identified genes exhibiting coordinated epigenetic and transcriptional changes, suggesting a role for DNA methylation in fine-tuning early defence-associated transcriptional responses. Together, these findings demonstrate that the effect of drought in delaying *V. longisporum* colonization is associated with specific regulation of detoxification processes, hormone signalling, and cell wall modification, coordinated by specific variations in epigenomic landscape.

4.3 Introduction

Climate change is increasing the frequency and severity of drought, exposing crop plants to environmental conditions in which multiple stresses act simultaneously to influence growth, development, and productivity (Seleiman et al., 2021). Drought represents one of the most significant abiotic constraints in agricultural systems, disrupting photosynthesis, impairing metabolic processes, and ultimately reducing crop yield and quality (Ahmar et al., 2019). In addition to abiotic limitations, crop plants are continually challenged by pathogens that further compromise performance. Among these, the vascular fungus *V. longisporum* poses a major threat to oilseed crops such as *B. napus*, where infection disrupts water and nutrient transport, leading to chlorosis, stunted growth, and yield losses (Depotter et al., 2016; Fradin and Thomma, 2006; Inderbitzin et al., 2011). Because both drought stress and vascular pathogen infection directly impact plant water relations and vascular function, their interaction represents a critical but underexplored aspect of plant stress biology (Suzuki et al., 2014).

Plant responses to combined abiotic and biotic stresses are increasingly recognised as complex and non-additive, involving intricate regulatory networks that integrate multiple signalling pathways (Suzuki et al., 2014). Rather than representing a simple summation of individual stress responses, combined stress conditions often result in unique physiological and molecular outcomes that cannot be predicted from single-stress studies. In the context of drought and *V. longisporum* infection, previous studies have reported contrasting effects, with some systems exhibiting enhanced resistance and others increased susceptibility (Bletsos et al., 1999). These contrasting responses highlight the importance of understanding how plants prioritize and coordinate defence, growth, and stress adaptation pathways under combined stress conditions.

Previous work in *B. napus* L. has exhibited that severe drought, i.e. 30% field capacity, reduces *V. longisporum* colonization, thereby decreasing disease severity (Kashyap et al., 2026). These

effects were linked to anatomical modifications, including the formation of suberized and lignified apoplastic barriers in roots and hypocotyls limiting pathogen movement through the vascular system. While these findings emphasize the importance of structural defence mechanisms, the molecular processes underpinning these events and other general defence mechanisms evoked by drought remain unknown.

It is well recognised that plant responses to stress are largely mediated by extensive transcriptional reprogramming involving genes associated with defence, hormone signalling, metabolism, and cellular structure (Jones and Dangl, 2006). Upon pathogen infection, plants activate a range of defence responses, including the production of antimicrobial compounds, and activation of signalling pathways mediated by SA, JA, and ET (Pieterse et al., 2012). These responses are often accompanied by the activation of detoxification mechanisms, such as glutathione-mediated redox regulation, which play a critical role in mitigating oxidative stress generated during pathogen attack (Noctor et al., 2014). Importantly, these pathways do not operate independently under combined stress conditions. Instead, drought can influence the timing, magnitude, and specificity of defence responses, leading to altered prioritization between growth and defence. For example, crosstalk between hormone signalling pathways, particularly involving ABA, JA, and ET, can modulate both abiotic stress tolerance and pathogen defence, resulting in coordinated yet context-dependent responses (Pieterse et al., 2012; Suzuki et al., 2014). Similarly, structural barriers such as cell wall reinforcement and suberin deposition may be differentially regulated under combined stress, contributing to enhanced resistance by limiting pathogen spread within host tissues.

In addition to transcriptional regulation, plant responses to stress are influenced by epigenetic mechanisms, including DNA methylation. DNA methylation, which occurs in CG, CHG, and CHH sequence contexts, also plays a role in regulating gene expression, genome stability, and transposable element activity (Law and Jacobsen, 2010; Zhang et al., 2018). Under stress conditions, dynamic changes in DNA methylation can modulate gene expression patterns, enabling plants to rapidly adapt to environmental challenges (Matzke and Mosher, 2014). Moreover, epigenetic regulation has been implicated in stress memory, allowing plants to exhibit enhanced or primed responses upon subsequent exposure to similar stresses (Crisp et al., 2016; Kinoshita and Seki, 2014; Lämke and Bäurle, 2017). Despite these advances, the role of epigenetic

mechanisms in coordinating plant responses to combined abiotic and biotic stresses remains poorly understood. In light of the above evidence, this study integrates transcriptomic and epigenetic analyses to investigate the molecular basis underlying the drought-mediated mitigation of *V. longisporum* colonization and disease progression in hypocotyls of *B. napus* during an early and late infection stage.

4.4 Materials and Methods

4.4.1 Plant material and growth conditions

Brassica napus cv. ‘Westar’ was used for all experiments. Seeds were sown in plastic trays (48 plants per tray) containing a commercial soilless substrate (Sunshine Mix #4; Sun Gro Horticulture, Canada), composed primarily of Canadian sphagnum peat moss (55-65%), coarse perlite, dolomitic limestone, and gypsum. Plants were grown under controlled environmental conditions with 16 h light/8 h dark photoperiod and day/night temperatures of 22/18 °C for 14 days, corresponding to the two-leaf stage of development, prior to inoculation.

4.4.2 Fungal culture

Verticillium longisporum strain UMB_1 (A1/D1 lineage) was cultured on potato dextrose agar (Sigma-Aldrich) supplemented with spectinomycin (100 µg mL⁻¹) and incubated at 25 °C in the dark for three weeks to promote conidial and microsclerotia development. For spore production, agar plugs from actively growing colonies were transferred to potato dextrose broth (Sigma-Aldrich) and incubated at 23 °C on a rotary shaker (120 rpm) for 7-14 days in darkness (Eynck et al., 2009).

Fungal spores were harvested by filtration through sterile muslin cloth to remove mycelial fragments. Glycerol stocks were prepared by mixing spore suspensions with sterile 20% (v/v) glycerol and stored at -80 °C for long-term preservation (Eynck et al., 2009). Spore concentrations were determined using a hemocytometer and adjusted to 5×10^5 conidia mL⁻¹ for *B. napus* inoculations.

4.4.3 Plant inoculation and imposition of water stress

Fourteen-day old *B. napus* seedlings were carefully uprooted, and roots were rinsed thoroughly with sterile distilled water to remove adhering substrate. Roots were trimmed to approximately 1-2 cm to facilitate infection, and seedlings were inoculated by root dipping in the *V. longisporum* conidial suspension (5×10^5 conidia mL⁻¹) for 30 min as described by Eynck et al., (2009). Control plants were mock inoculated with sterile distilled water. Following inoculation, seedlings were transplanted into fresh soil and maintained under the same growth conditions for 3 days prior to imposition of water stress.

Plants were grown under either water control conditions: control (C), i.e. field capacity, determined by saturating the pots filled with the soil medium, allowing drainage for 2 h, or drought (D), i.e. 30% field capacity (Kashyap et al., 2026). Water content in the soil was monitored daily using Theta Probe (Delta-T Devices, UK). The experiment consisted of four treatments: *V. longisporum*-inoculated plants grown under water control (C+) or drought (D+) conditions, and mock-inoculated plants grown under water control (C-) or drought (D-) conditions.

4.4.4 Tissue sampling for transcriptomic and epigenetic analyses

To capture transcriptional responses to combined drought and pathogen stress, hypocotyl tissues (C+, D+, C- and D-) were collected at 2 and 4 days after imposition of the water regime (DAWR). These time points correspond to early (2 DAWR) and late (4 DAWR) infection of the hypocotyl (Kashyap et al., 2026). Given the high costs associated with the epigenetic analyses, DNA methylation studies were only conducted to compare the effect of early *V. longisporum* infection (2 DAWR) during drought (D+ vs. D-), known to delay disease progression and fungal colonization (Kashyap et al., 2026). Unless otherwise indicated, analyses were conducted using three biological replicates, each consisting of hypocotyls pooled from 10 plants.

4.4.5 RNA extraction and sequencing

Total RNA was extracted from frozen hypocotyl tissue stored at -80 °C using the RNeasy Plant Mini Kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's instructions, including an on-column DNase I digestion step to remove residual genomic DNA. Prior to extraction, tissues were ground to a fine powder in liquid nitrogen using a mortar and pestle to ensure efficient RNA recovery. RNA purity was assessed using a NanoDrop spectrophotometer (Thermo Scientific) by measuring A260/280 and A260/230 ratios, and RNA concentration was quantified using a Qubit

fluorometer (Thermo Fisher Scientific). RNA integrity was evaluated using an Agilent TapeStation system, and only samples with an RNA integrity number (RIN) ≥ 7 were used for downstream analyses (Schroeder et al., 2006).

RNA sequencing libraries were prepared using a stranded, rRNA-depleted plant RNA library preparation protocol. Sequencing was conducted on an Illumina NovaSeq platform using paired-end 100 bp reads (PE100), with a target depth of ~ 40 million reads per sample (Bentley et al., 2008).

4.4.6 RNA-seq data processing and differential expression analysis

All computational analyses were performed using high-performance computing resources provided by the Digital Research Alliance of Canada. Raw sequencing read quality was assessed using FastQC v0.11.9 (Andrews, 2010) to evaluate sequencing quality parameters and ensure the reliability of downstream analyses. High-quality paired end reads (i.e., reads with $\geq 80\%$ bases having Phred quality scores ≥ 30 , minimal adapter contamination, and $< 5\%$ ambiguous bases) were aligned to the *B. napus* reference genome BnIR Westar.v0 using HISAT2 v2.2.1 (Kim et al., 2019) with default parameters, allowing spliced alignment and reporting primary alignments. Gene-level read counts were generated using featureCounts v2.0.3 (Liao et al., 2014) with default settings, assigning reads to exons based on the reference annotation and summarizing counts at the gene level. Differential gene expression analysis was conducted in R using the DESeq2 package (Love et al., 2014). A count matrix of gene-level read counts was used to construct a DESeqDataSet with a design formula incorporating treatment and time point effects. Size factor normalization and dispersion estimation were performed using default parameters, followed by negative binomial generalized linear model fitting to identify Differentially expressed genes (DEGs). Log₂ fold change shrinkage was applied using the *ashr* method (Stephens, 2017). Genes were considered differentially expressed at a false discovery rate (FDR) < 0.05 (Benjamini-Hochberg correction) with an absolute log₂ fold change ≥ 2 . To facilitate functional interpretation, *B. napus* gene sequences were aligned against *A. thaliana* gene models from TAIR10 using BLASTn/BLASTp (Altschul et al., 1990; Camacho et al., 2009) to identify putative orthologs. Functional annotations and gene descriptions were inferred based on corresponding *A. thaliana* gene information. Principal component analysis (PCA) and data visualization were performed using ggplot2 (Wickham, 2011). Three pairwise contrasts were analyzed at both time points (2 and 4 DAWR):

C+ vs. C−, D+ vs. D−, and D− vs. C−. Gene ontology (GO) enrichment analysis was conducted separately for upregulated and downregulated gene sets using the annotation provided with the BnIR Westar.v0 reference dataset (Ashburner et al., 2000; The Gene Ontology Consortium, 2021).

4.4.7 Gene co-expression and regulatory network analysis

To explore higher order regulatory relationships beyond differential expression, gene co-expression network (GCN) analysis was performed using the BioNERO R package (Almeida-Silva and Venancio, 2022). TPM-normalized expression matrices were used as input. Outlier samples were identified using ZK filtering, and principal component (PC) correction was applied to reduce unwanted technical variation. A signed hybrid network was constructed based on the Weighted Gene Co-Expression Network Analysis (WGCNA) framework implemented in BioNERO. The soft-thresholding power (β) was determined using the scale-free topology criterion ($R^2 \geq 0.8$). Gene modules were detected by hierarchical clustering with dynamic tree cutting and subsequently merged using a module eigengene correlation threshold of 0.8. Module-trait associations were assessed using Pearson correlation between module eigengenes and experimental traits. Additionally, gene regulatory networks (GRNs) were inferred using a consensus-based approach implemented in BioNERO, integrating GENIE3 (random forest-based), ARACNE (mutual information-based), and CLR algorithms. A curated list of transcription factors (TFs) was identified from the *B. napus* genome using the iTAK software (Zheng et al., 2016) and used as candidate regulators for GRN inference. GRN hubs were defined as the top 10% most highly connected regulators based on network degree. Network visualization and topological analyses were performed using Cytoscape (v3.10.1).

4.4.8 Quantitative real-time PCR (qRT-PCR) for validation analyses

For validation of RNA-seq results, total RNA was reverse transcribed into cDNA using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA, USA). *ACTIN 2* (ACT2; *BnaA10G0010600WE*) was used as the internal reference gene. Gene-specific primers were designed using NCBI Primer-BLAST and verified for specificity against the *B. napus* genome. Relative transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

4.4.9 Microscopic analyses

General histological analyses were carried out by thin sectioning using Toluidine Blue O to visualize general structural features. Lignin and suberin were localized using phloroglucinol-HCl and Fluorol Yellow 088 respectively (Kashyap et al., 2026). Fungal detection was performed using Wheat Germ Agglutinin (WGA) conjugated with Alexa Fluor® 488, following counterstaining with propidium iodide to visualize cell walls. 2',7'-dichlorodihydrofluorescein diacetate was utilized to detect ROS in hypocotyl tissue during infection (Yokawa et al., 2011), while reduced glutathione (GSH) was visualized with Monochlorobimane following the procedure in Das et al. (2018).

4.4.10 Glutathione measurements

Spectrophotometric measurements of reduced (GSH) or oxidized glutathione (GSSG) were carried out exactly as described by Zhang and Kirkham (1996).

4.4.11 High-molecular-weight DNA extraction for epigenetic analysis

To complement transcriptomic analyses, genome-wide DNA methylation profiling was performed using ONT sequencing. To enable genome-wide DNA methylation analysis under drought and pathogen stress conditions, genomic DNA was extracted from frozen hypocotyl tissue following an optimized protocol suitable for long-read sequencing (Schalamun Miriam & Schalamun Benjamin, 2017). Plant material was finely powdered in liquid nitrogen prior to extraction. Samples were incubated in a buffer system formulated to limit oxidative damage and reduce co-isolation of secondary metabolites. DNA purification was carried out using bead-based clean-up procedures with minimal physical disturbance to preserve long DNA fragments and subsequently subjected to controlled shearing using Covaris g-TUBEs to obtain a uniform fragment size distribution, followed by size selection using a BluePippin system to enrich for longer DNA fragments suitable for ONT sequencing. DNA yield and purity were evaluated using NanoDrop spectrophotometry and Qubit fluorometric quantification. Fragment size distribution and integrity were assessed by agarose gel electrophoresis and Agilent TapeStation analysis. Only DNA samples meeting quality requirements were used for downstream library preparation.

4.4.12 Library preparation for Oxford Nanopore Technologies sequencing

Sequencing libraries were constructed using the ONT ligation-based kit with native barcoding (SQK-NBD114.24) according to the manufacturer's protocol. DNA repair and end-preparation reactions were followed by barcode ligation and adapter attachment, with bead purification steps performed between each stage. No amplification step was included during library preparation to ensure retention of native DNA methylation signatures.

4.4.13 Oxford Nanopore Technologies sequencing, basecalling, read alignment, and methylation detection

Sequencing was carried out using the ONT P2 Solo platform equipped with R10.4.1 flow cells in accordance with standard operating procedures. Biological replicates representing each treatment group (D⁺ and D[−]) at 2 DAWR were multiplexed and loaded onto a single flow cell. Data acquisition was continued for up to 72 hours or until a noticeable reduction in active sequencing pores was observed. Raw electrical signal data were processed using Dorado basecalling software v7.9.8 with a high-accuracy model and modified base detection enabled for cytosine methylation. Only reads meeting default quality criteria were retained for subsequent analyses. Basecalled reads were aligned to the *B. napus* Westar reference genome using minimap2 with parameters optimized for long-read data (Li, 2018). The resulting alignments were converted, sorted, and indexed using samtools (Li et al., 2009).

Methylation information was extracted from aligned reads using modkit (Oxford Nanopore Technologies, 2023), producing genome-wide per-base methylation profiles across CG, CHG, and CHH sequence contexts.

4.4.14 Differential methylation analysis

Methylation data were processed independently for CG, CHG, and CHH contexts. For regional analysis, per-base methylation calls were aggregated into non-overlapping 1 kb genomic windows. Cytosine sites with a minimum sequencing depth of five reads were retained for downstream analysis. Comparative methylation analysis between treatment groups (D⁺ vs. D[−]) was conducted using the DSS package in R, which employs a beta-binomial statistical framework to detect differential methylation (Feng and Wu, 2019). Replicate-level data were used to construct BSseq

objects, and context-specific coverage thresholds were applied ($CG \geq 200$, $CHG \geq 100$, $CHH \geq 50$) to account for differences in cytosine density and methylation patterns across contexts.

DML were defined as individual cytosine positions showing statistically significant differences between treatments. DMR were subsequently identified as contiguous genomic segments containing multiple significant loci. Regions were categorized as hypermethylated or hypomethylated based on the direction of methylation change in D+ relative to D- samples.

4.4.15 Annotation of differentially methylated loci and integration with transcriptomics

Differentially methylated loci were annotated by intersecting genomic coordinates with gene features using BED Tools (Quinlan and Hall, 2010), based on the *B. napus* reference genome annotation (Yang et al., 2023). DML overlapping gene bodies were classified as genic, while those located within ± 2 kb of annotated genes was considered putative regulatory regions and assigned to the nearest gene.

To investigate the relationship between DNA methylation and transcriptional regulation, DML-associated genes were integrated with DEGs identified from RNA-seq analysis under the same treatment conditions (D+ vs. D-). This approach enabled the identification of genes exhibiting coordinated epigenetic and transcriptional changes during early infection. To further assess regulatory patterns, DML were categorized based on methylation direction (hypermethylated or hypomethylated in D+ relative to D-), and their association with gene expression changes (upregulated or downregulated DEGs) was examined. This allowed evaluation of potential relationships between methylation state and transcriptional activity, particularly within gene bodies and proximal regulatory regions.

Functional characterization of DML-DEG overlapping genes was performed using *A. thaliana* ortholog annotations (Altschul et al., 1990; Camacho et al., 2009), enabling the identification of biological processes enriched among genes potentially regulated by DNA methylation. Given that DNA methylation profiling was conducted at the early infection stage (2 DAWR), this integrative analysis was designed to capture primary epigenetic regulatory events associated with downstream transcriptional responses under combined drought and pathogen stress.

4.5 Results

4.5.1 Sequencing overview of transcriptomic and epigenomic datasets

To characterize the molecular responses of *B. napus* to *V. longisporum* infection under contrasting water regimes, transcriptomic and epigenomic datasets were generated from hypocotyl tissues. For transcriptome profiling, RNA sequencing was performed on 24 libraries representing four treatment combinations (C–, C+, D–, and D+) collected at 2 and 4 DAWR with three biological replicates per treatment. Libraries were prepared using an rRNA-depleted plant RNA protocol and sequenced on an Illumina NovaSeq platform using paired-end 100 bp reads. Sequencing generated approximately 1.53 billion paired end reads across all libraries, corresponding to an average of 63.6 million reads per sample. On average, 80.82% of reads aligned to the *B. napus* Westar reference genome, with 60.19% mapping uniquely, indicating high-quality transcriptome data suitable for downstream analyses.

For epigenomic profiling, ONT long-read sequencing was performed on six hypocotyl samples representing the D– and D+ treatments collected at 2 DAWR, with three biological replicates per treatment. D– samples generated an average of 23.9 Gb of passed sequence data per replicate, corresponding to a mean genome coverage of 23.7×, whereas D+ samples generated an average of 46.8 Gb per replicate with a mean coverage of 46.5×. Read length distributions were comparable among samples, with average read N50 value of approximately 10 kb for the D– and D+ treatments both. Sequencing depth and genome coverage were sufficient to support robust genome-wide methylation profiling and downstream differential methylation analyses.

Principal component analysis and hierarchical clustering of the RNA-seq dataset revealed strong grouping of biological replicates and clear separation among treatment groups, demonstrating distinct transcriptional responses to drought stress and *V. longisporum* infection (Figure 1A–C).

4.5.2 Changes in global expression patterns evoked by *V. longisporum* under different water regimes

We have previously reported that drought (D), i.e. 30% field capacity, decreases *V. longisporum* colonization and disease progression in *B. napus* plants (Kashyap et al., 2026). The acropetal

progression of the fungus, which in well-watered control (C) plants occurs uniformly through roots, hypocotyls and leaves, is delayed under drought conditions. Drought stress hinders the spread of the pathogen reducing fungal load in the hypocotyl vasculature (Kashyap et al., 2026). These results suggest that physiological/molecular responses in hypocotyl cells, might contribute to restrict colonization. To identify hypocotyl-specific transcriptomic changes elicited by the fungus, we profiled global gene expression patterns in hypocotyls of mock-inoculated (–) or *V. longisporum*, inoculated (+) plants grown under field capacity (control, C) or 30% field capacity (drought, D) at 2 and 4 days after imposition of the water regimes (DAWR). These specific time points correspond to two infection stages: an early stage defined by the fungal colonization of the root (2 DAWR), and a late stage demarked by the spread of the pathogen to the hypocotyl (4 DAWR) (Kashyap et al., 2026).

Three pairwise comparisons were performed to examine the effect of *V. longisporum* under control (C+ vs. C–), or drought (D+ vs. D–) conditions, and the effect of drought alone (D– vs. C–) at 2 and 4 DAWR. DEGs were identified using a false discovery rate (FDR) < 0.05 and an absolute log₂ fold change ≥ 2. Validation on 9 selected genes revealed similar expression patterns (up or downregulation) between qRT-PCR and RNA-seq analyses (Supplemental Figure S4.1). Dissimilarity matrix analysis (Figure 4.1A), PCA (Figure 4.1B), and clustering of the top 2,000 most variable genes (Figure 4.1C) revealed a clear separation of samples based on infection status and water regime. Infection resulted in a pronounced shift in gene expression that intensified from 2 to 4 DAWR (Figure 4.1C).

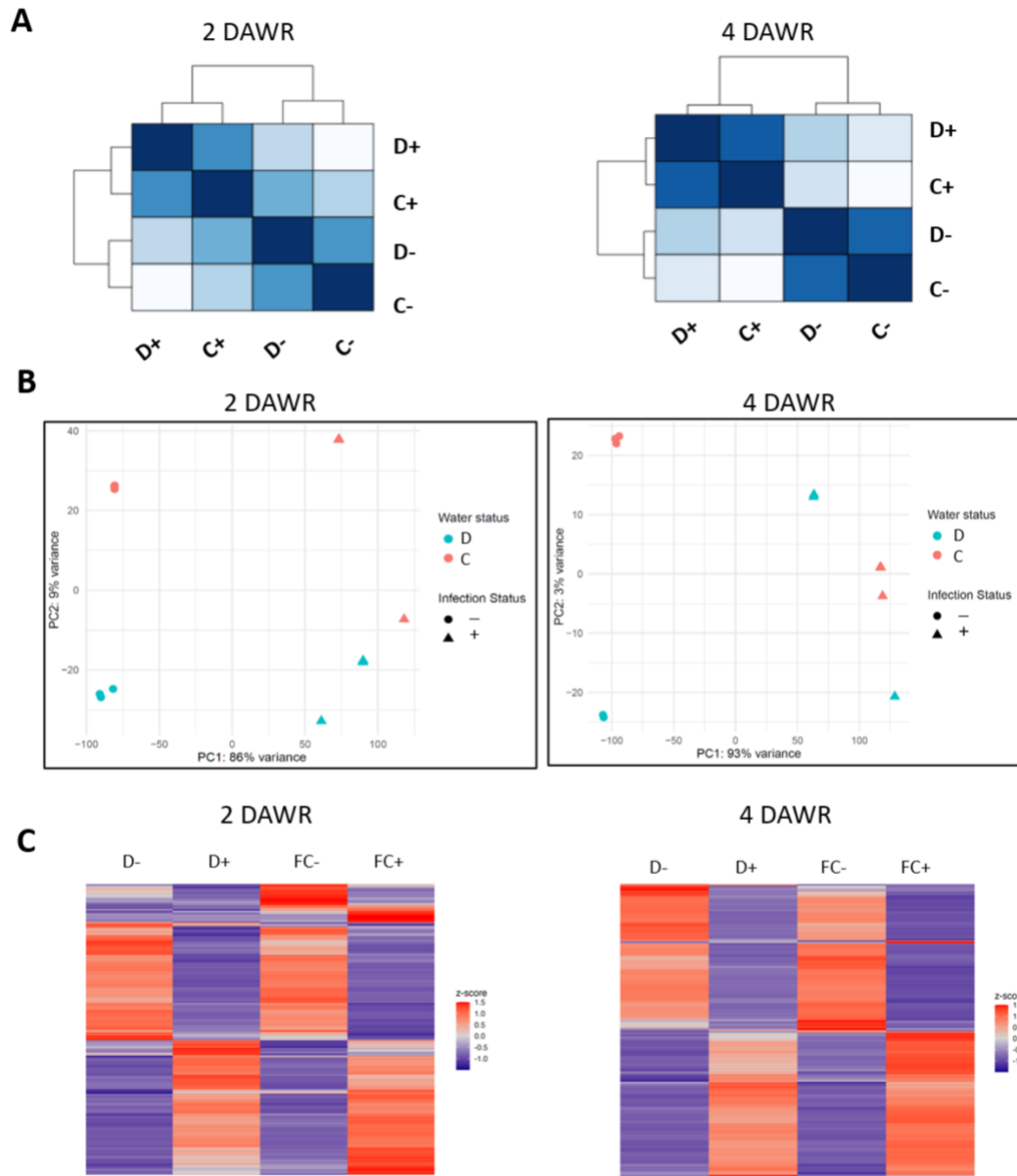
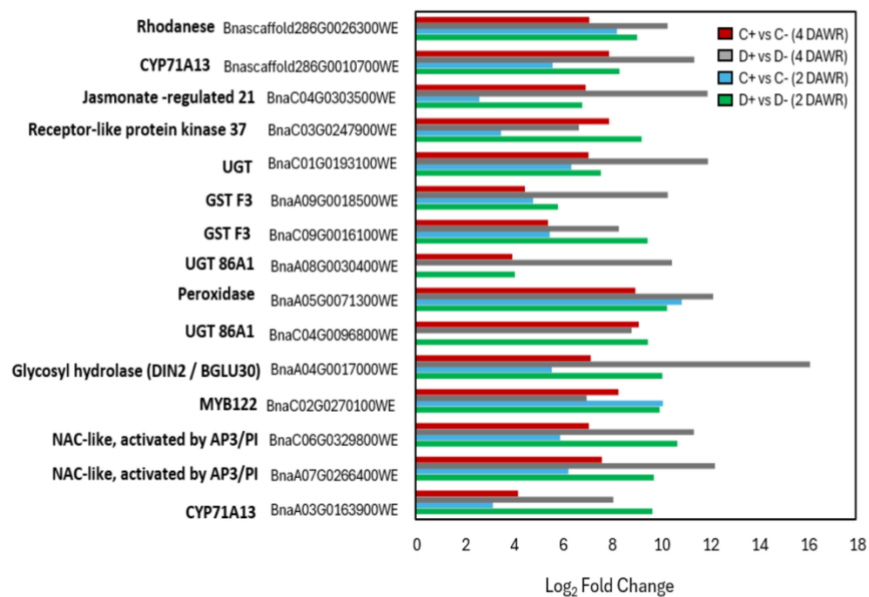


Figure 4.1 A. Dissimilarity matrix among the different treatments (D+, D-, C+, and C-) at 2 and 4 DAWR, Colour value corresponds to Euclidean distance between samples; counts normalized to library size were used as input for computing distance. A darker blue denotes higher similarity between samples. B. Principal component analyses of differentially expressed genes among treatments. C. Hierarchically clustered heatmap of the top 2,000 most variable genes across treatments at 2 and 4 DAWR, showing z-score-scaled expression patterns at (FDR < 0.05).

Transcripts preferentially induced by the fungus under drought (D) included *CYTOCHROME 71 POLYPEPTIDE A 13* (*CYP71A13*), several *GLUTATHIONE S-TRANSFERASES* (*GST*), *UDP-GLYCOSYLTRANSFERASES* (*UGT*), and two *NAC*-like transcription factors (Figure 4.2A). In contrast, transcripts encoding key glucosinolate biosynthetic enzymes, *CYP79F1* and *ISOPROPYLMALATE ISOMERASE 2* (*IPM2*), were preferentially suppressed by *V. longisporum* under control conditions at 4 DAWR. A similar expression pattern was observed for *MYB29*, a regulator of ion homeostasis (Figure 4.2B).

A



B

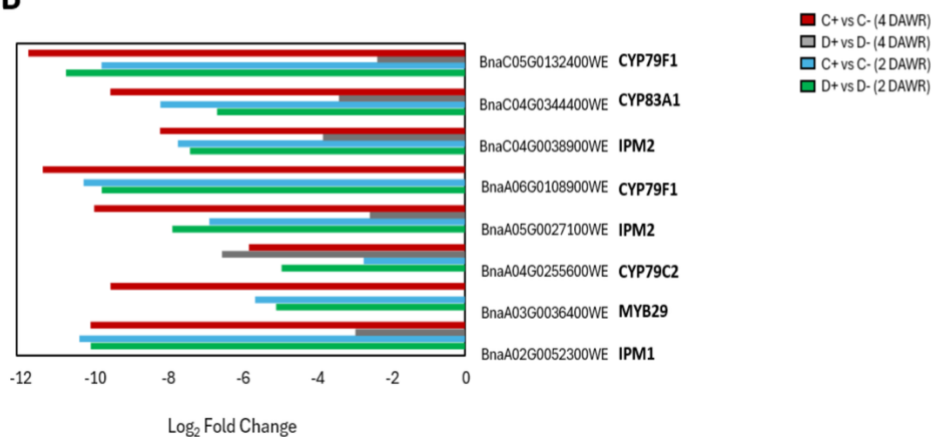


Figure 4.2 Exemplary DEGs selected from the top 20 most deregulated genes among the treatments. The graphs show the expression of genes upregulated in response to *Verticillium longisporum* under Drought (A) and downregulated in response to *V. longisporum* under Control (B) at 2 and 4 Days after water regime (DAWR).

4.5.3 Functional enrichment analysis

At 2 DAWR, corresponding to extensive fungal colonization of root tissue, a total of 1,128 and 1,372 unique DEG were upregulated by *V. longisporum* in C or D hypocotyls (Figure 4.3A). GO analyses of induced genes under C conditions (C+ vs. C-) revealed an enrichment associated with general and diverse developmental processes, as well as hormones, especially gibberellins and auxin, and metabolic events centred around carbohydrates, phyloquinones, and several secondary metabolites (Figure 4.3B). GO terms of unique DEG induced by the fungus in D hypocotyls (D+ vs. D-) were heavily represented by biological processes pertaining to defence and detoxification. Responses to pathogens, systemic acquired resistance, as well as biosynthesis of glucosinolates and metabolism of glutathione were among the most enriched categories (Figure 4.3B). Processes associated with ET synthesis and metabolism were also represented. Unique DEG (1,275) downregulated by *V. longisporum* in C plants were enriched in GO terms related to cell wall modification and reinforcement, with a heavy representation of DEG involved in secondary wall formation, xylan and lignin synthesis and metabolism (Figure 4.3C). The most representative GO categories of the 1,511 unique DEG downregulated by *V. longisporum* in D plants were clustered into processes pertaining to cell cycle/division and kinase binding and activity (Figure 4.3C).

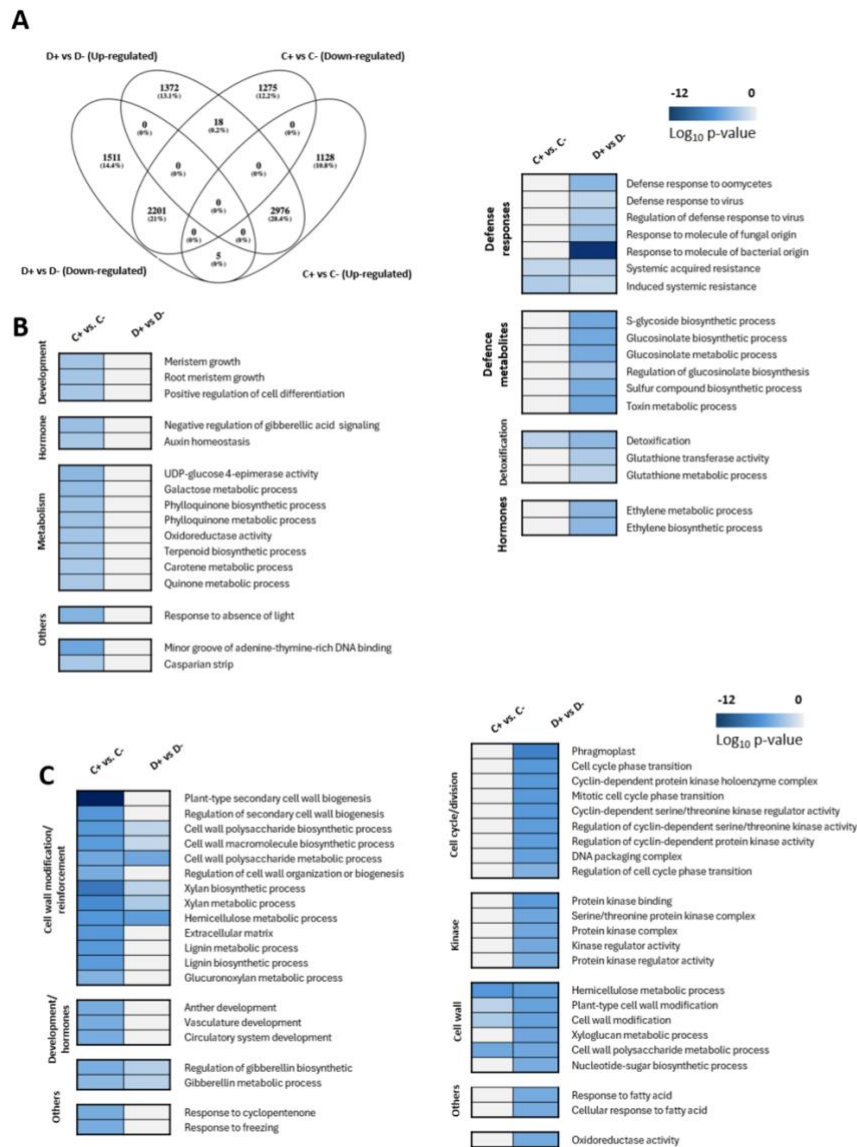


Figure 4.3 Differentially expressed genes and GO term enrichment 2 Days after water regime (DAWR). A. Venn diagram showing significantly deregulated DEG (FDR < 0.05) among the different treatments. B. Heat maps of significantly enriched GO terms (FDR < 0.05) composed by unique DEG upregulated by *Verticillium longisporum* under C (left map) or D (right map) conditions. C. Heat maps of significantly enriched GO terms (FDR < 0.05) composed of unique DEG downregulated by *Verticillium longisporum* under C (left map) or D (right map) conditions.

At 4 DAWR, characterized by the spread of the fungus into the hypocotyls (Kashyap et al., 2026), we identified 1,874 and 1,444 DEG up regulated by the fungus and unique to C or D conditions (Figure 4.4A). *Verticillium*-induced DEG in C plants (C⁺ vs. C⁻) were mainly enriched in processes related to light responses, defence/stress responses, ranging from systemic acquired resistance to resistance to insects, and hormones (Figure 4.4B). In D plants, the upregulated DEG were heavily involved in detoxification processes, with significant representation of GO categories related to glutathione metabolism and respiratory burst. Processes pertaining to membrane modifications and specifically linked to calcium transport were also enriched. *V. longisporum* infection also decreased the expression of 3,340 and 641 DEG, unique to C or D conditions (Figure 4.4A). Heavily enriched categories of downregulated DEG in infected C hypocotyls (C⁺ vs. C⁻) were primarily linked to biological processes regulating cell cycle events specific to DNA replication and repair (Figure 4.4C). This pattern contrasted with infected D hypocotyls (D⁺ vs. D⁻) characterized by suppression of genes participating in redox processes, hormone responses specific to brassinosteroids and cytokinin, as well as events linked to development and secondary metabolism (Figure 4.4C).

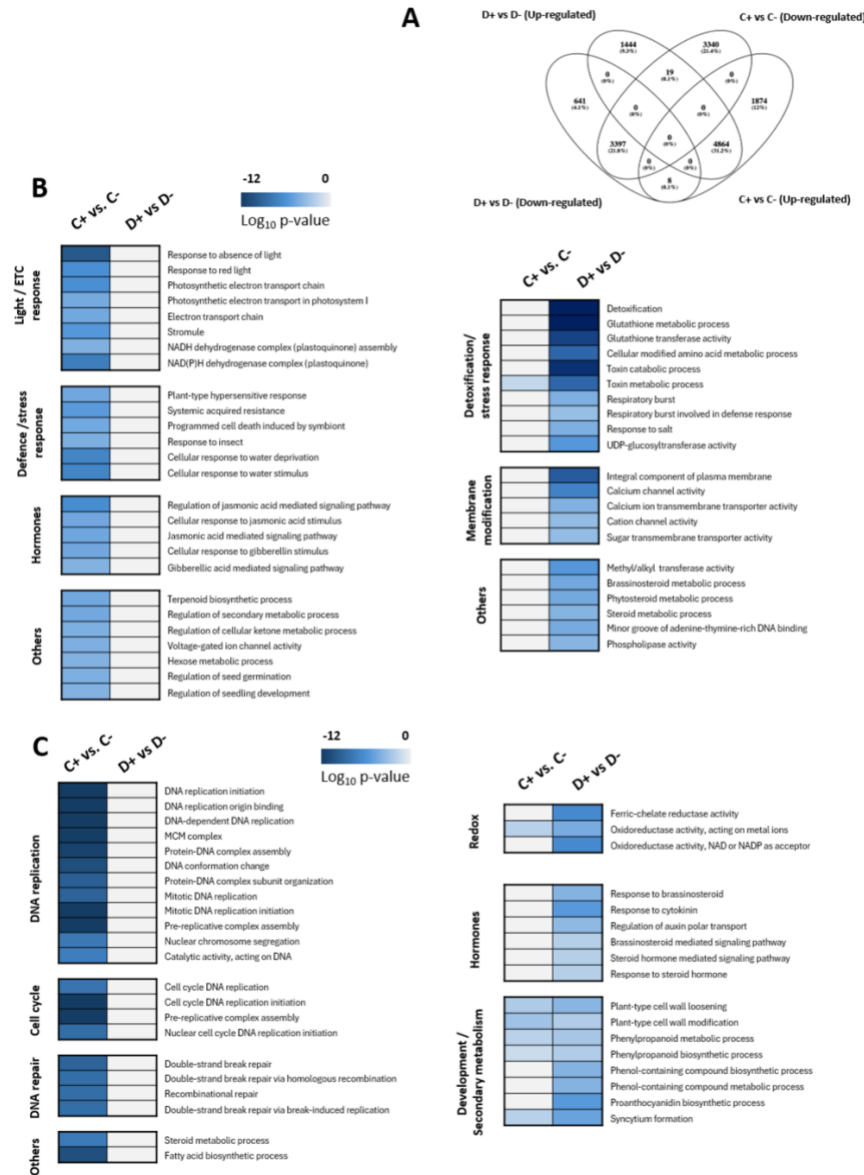


Figure 4.4 Differentially expressed genes and GO term enrichment 4 Days after water regime. (A) Venn diagram showing significantly deregulated DEG (FDR < 0.05) among the different treatments. (B) Heat maps of significantly enriched GO terms (FDR < 0.05) composed by unique DEG upregulated by *Verticillium* under C (left map) or D (right map) conditions. (C) Heat maps of significantly enriched GO terms (FDR < 0.05) composed of unique DEG downregulated by *Verticillium* under C (left map) or D (right map) conditions.

4.5.4 Drought activates unique defence and detoxification mechanisms in *V. longisporum* inoculated plants

To unveil some of the molecular mechanisms elicited by drought to delay disease progression and fungal colonization, we extracted DEG from the most enriched GO categories at the early (2 DAWR) or late (4 DAWR) infection stages. First, we analyzed the GO categories pertaining to defence and detoxification processes, which were highly activated in infected plants by drought (Figure 4.3A and 4.4A). Relative to hypocotyl of well-watered plants, several DEG related to synthesis or modifications of glucosinolates (GLS), components of canonical responses to pathogen challenge, were more consistently upregulated by *V. longisporum* especially during early infection (2 DAWR) in hypocotyls of drought plants (compare C+ vs. C- with D+ vs. D- in Figure 4.5A). These included *APS KINASE 2*, *SULFOTRANSFERSE 17*, and several types *CYTOCHROME 450 (CYP)* members such as *CYP79B3*, *CYP81F2*, *CYP81H1*, and *CYP81K1*. A drought specific induction during both early (2 DAWR) and late (4 DAWR) infection stages was also noted for several *CHITINASES* and *PROTEINASES (CP)*, *GLYCOSYL HYDROLASES* and *TRANSFERASES*, as well as components of H₂O₂ detoxification systems, and defence-related transcription factors (Figure 4.5A). The latest comprised several *NAC* and *WRKY*, and *MYB* members, including *MYB108* which is known to confer resistance to *Verticillium* species in *B. rapa* (Su et al., 2023). While some of these DEGs were also upregulated in response to *V. longisporum* infection in C hypocotyls (C+ vs. C-), especially those encoding regulators of glycosyl modification and transcription factors, their induction was delayed and became more apparent at 4 DAWR, indicating a delayed plant response to pathogen infection. Relative to control conditions, inoculation of water-stressed hypocotyls also resulted in a more consistent up regulation of many DEG related to general defence mechanisms. They included a variety of disease resistant proteins, *PATHOGENESIS-RELATED 4*, a *RHODANSASE/PHOSPHATASE*, and a *PEROXIDASE*. Induction of many of these DEG was more apparent during the later infection stage (4 DAWR). A similar trend also occurred for many DEG linked to glutathione metabolism, especially at 4 DAWR; they comprised a variety of *GLUTATHIONE S-TRANSFERASES* of the *F3* and *TAU* sub-families (Figure 4.5A).

The preferential induction of DEG related to H₂O₂ detoxification processes and glutathione metabolism in response to *V. longisporum* under drought (compare C+ vs. C- with D+ vs. D- in

Figure 4.5A) suggests a differential accumulation of ROS and glutathione, implicated in ROS detoxification (Noctor et al., 2014), between C⁺ and D⁺ tissue. This was tested by first localizing ROS with 2',7'-dichlorodihydrofluorescein diacetate and reduced glutathione (GSH) with monochlorobimane and then quantifying glutathione in C⁺ and D⁺ hypocotyls. Major differences in ROS staining patterns between C⁺ and D⁺ hypocotyls were observed in isolated cortical cells, which exhibited intense fluorescence in C⁺ (arrows in Figure 4.5Ba, c and d) but not in D⁺ (Figure 4.5Bb and e). An opposite trend was noted for GSH staining. Cortical cells of C⁺ hypocotyls were almost devoid of signal (Figure 4.5Ca and e) while intense staining occurred in the cortex of D⁺ (arrows in Figure 4.5Cb, c and d). The levels of both reduced (GSH) and oxidized (GSSG) glutathione were higher in hypocotyls of D⁺ plants relative to their C⁺ counterparts (Figure 4.5D).

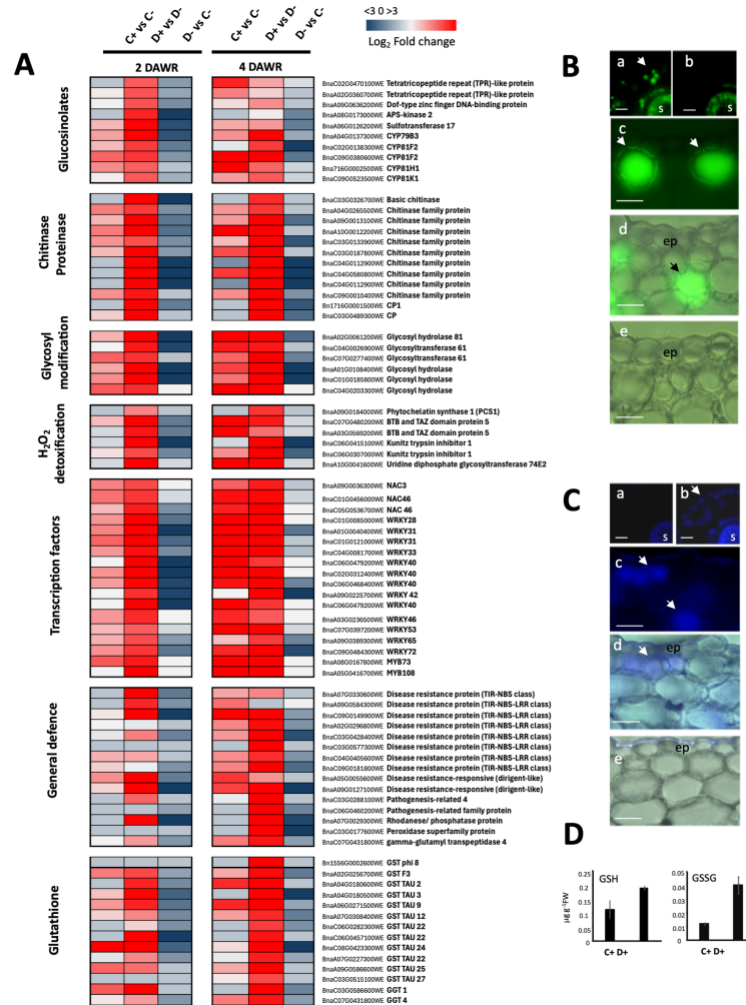


Figure 4.5 A. Heat map of significant DEG (FDR < 0.05) belonging to the GO categories related to overall defence and detoxification mechanisms. The map compares the expression of exemplary genes among the different treatments (C+ vs. C-, D+ vs. D-, and D- vs. C-) at 2 and 4 DAWR. B. Localization of ROS in C+ (Figure a, c, and d) and D+ (b and e) hypocotyls. Arrows denote the preferential ROS staining in cortical cells of C+ hypocotyls. s stele; ep, epidermis. Scale bars= 110 μ m (a, b) and 25 μ m (c-e). C. Localization of GSH in C+ (Figure a and e) and D+ (b, c and d) hypocotyls. Arrows denote the preferential GSH staining in cortical cells of D+ hypocotyls. s stele; ep, epidermis. Scale bars= 110 μ m (a, b) and 25 μ m (c-e). D. Measurements of reduced (GSH) and oxidized (GSSG) glutathione in hypocotyls of C+ and D+ plants. Values $\pm$ SE are means of three biological replicates each containing hypocotyls pooled from 10 plants. Letters on bars denote statistical differences (p<0.05).

Next, we analyzed DEG related to defence / stress response processes that were preferentially induced in response to *V. longisporum* under control water regimes (C+ vs. C–) relative to drought (D+ vs. D–) (Figure 4.6). Many of these DEG were involved in systemic acquired resistance (SAR), as well as cell death and hypersensitive response (HR). Exemplary DEG include several *ADC SYNTHASES*, *JASONATE-ZIM-DOMAIN* (JAZ) 10, two *NAC* 89, and a *MAP KINASE* 5. Their induction by the fungus in well-watered hypocotyls was more consistent at 4 DAWR.

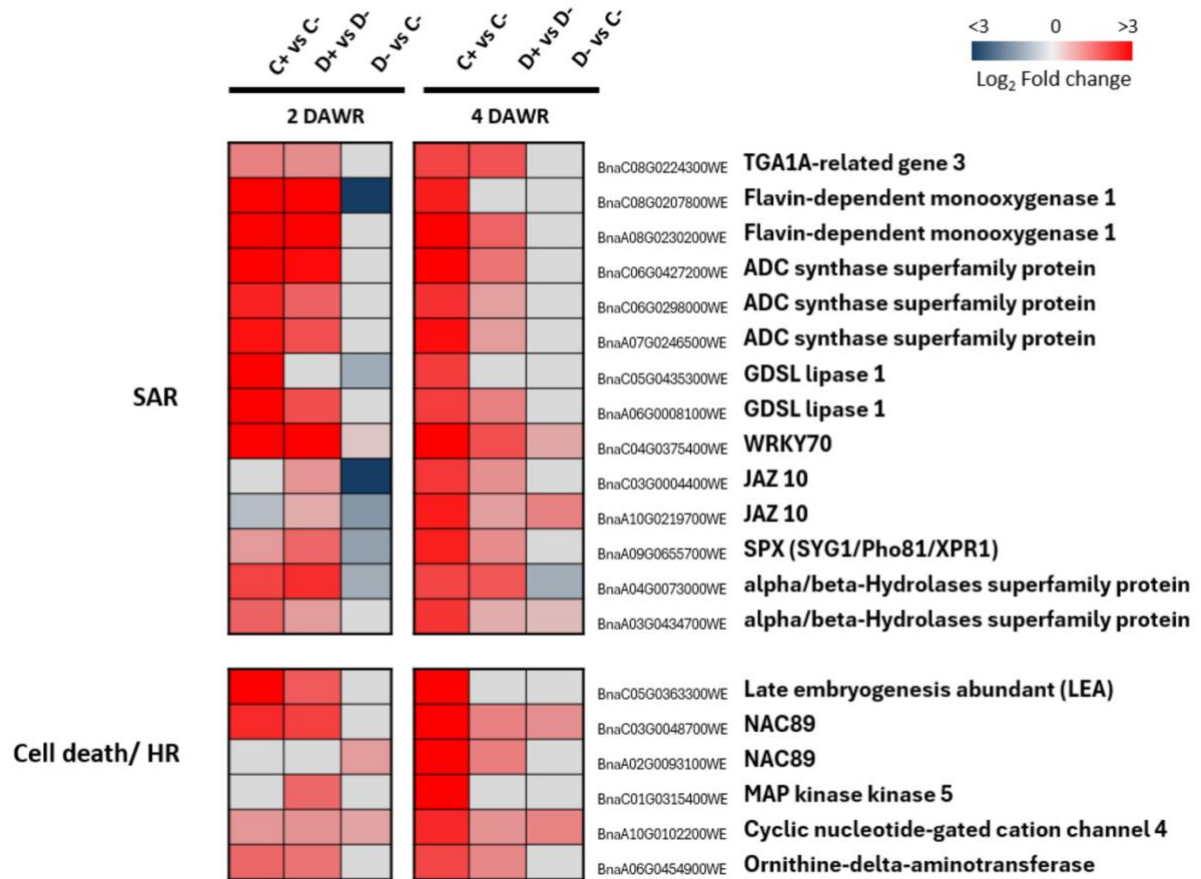


Figure 4.6 Heat map of significant DEG (FDR < 0.05) belonging to the GO categories related to systemic acquired resistance (SAR) and cell death/hypersensitive response (HR). The map compares the expression of example genes among the different treatments (C+ vs. C-, D+ vs. D-, and D- vs. C-) at 2 and 4 Days after water regime (DAWR)

4.5.5 *Verticillium*-induced alteration of cell wall components are influenced by drought

Cell wall modifications and reinforcement are among the biological processes most repressed in response to *V. longisporum* in hypocotyls of well-watered plants (C+ vs. C– in Figure 4.3C). DEG within these categories included components of secondary cell wall organization, which were downregulated at both infection stages regardless of the water regime (Figure 4.7A). Drought, however, attenuated this suppression during the early infection stage (compare C+ vs. C– with D+ vs. D– at 2 DAWR). The most pronounced differences between water regimes at 2 DAWR were noted for *MYB26*, one *COBRA-like*, and several DEG encoding laccase/diphenol oxidases and cellulose synthases (CES). At 4 DAWR *NAC84* was heavily induced in D+ vs. D– while the expression of two DEG encoding CES was unchanged by the pathogen under drought, despite a suppression in C+ vs. C–.

Next, we analyzed DEG involved in lignin synthesis and modification. Compared with control conditions, drought reduced the suppressive effect of *Verticillium* on the expression of several genes at 2 or 4 DAWR. They included *CINNAMYL ALCHOL DEHYDROGENASE 8 (CAD8)*, *CAFFEYL-CoA-METHYLTRANSFERASE 1 (COoAMT1)*, *NAC12*, and *MYB 63*. The transcript abundance of other genes, including *LACCASE 7* and *13*, *CINNAMATE-4-HYDROXYLASE (C4H)*, and *CINNAMYL ALCOHOL DEHYDROGENASE 5 (CAD5)* were preferentially induced in D+ vs. D– at both infection stages, relative to C+ vs. C–. Plants under drought and inoculated with *V. longisporum* also exhibited an increased transcript abundance of two key regulators of suberin synthesis, *CY77A6* implicated in suberin monomer biosynthesis and *MYB41* a regulator of suberin deposition; the expression of the former was only marginally increased by the fungus in hypocotyls of control plants (C+ vs. C–) (Figure 4.7A). Genes involved in the synthesis of hemicellulose and xylans, which contribute to secondary wall architecture and structural organization were consistently downregulated in response to *V. longisporum* under control conditions (C+ vs. C–) at both 2 and 4 DAWR. They included *XYLOSYLTRANSFERASE 1 (XYLT1)*, *NUCLEOTIDE-DIPHOSPHO-SUGAR TRANSFERASES*, and several plant glycogenin-like starch initiation proteins (PGSIP). Imposition of drought mitigated their suppression (D+ vs. D–). Relative to control water conditions, infection of *V. longisporum* in water stressed tissue also increased the transcripts levels of many *INVERTASES* and *XYLOGLUCAN ENDOTRANSGLUCOSYLASES (XTH 11, 21, 29 and 30)* especially at 4 DAWR. In the absence

of *Verticillium*, the effect of drought on the expression of cell wall related DEG was generally moderate; most pronounced suppression was observed for *NAC 84*, *MYB15*, several *LACCASE 7*, two *INVERTASES* and *XTH 29* (Figure 4.7A).

The induction of many DEG linked to lignin and suberin following *V. longisporum* inoculation of drought plants (Figure 4.7A), prompted us to localize these two wall components in hypocotyl tissue sectioned 0.5 cm above the root-hypocotyl junction at 4 DAWR, confirming our previous studies (Kashyap et al., 2026). D+ hypocotyls exhibited the precocious formation of the periderm (arrow in Figure 4.7B, b) staining positively with Berberine-hemisulfate, detecting lignified cell walls (arrow in Figure 4.7B, d), and with Phloroglucinol-HCl and Fluorol Yellow 088 which more specifically identify cells containing lignin (asterisks in Figure 4.7B, f) and suberin (asterisks in Figure 4.7B, h) respectively. Very faint staining occurred in infected hypocotyls of well-watered plants (C+) (Figure 4.7B, c, e, and g). In D+ hypocotyls, the precocious formation of the periderm along with the accumulation of lignin and suberin, likely act as a physical barrier for the spread of the pathogen, as denoted by the accumulation of fungal hyphae on the outer peridermal cells (Figure 4.7B, j). This contrasted with C+ hypocotyls where the fungal growth often extended in the subtending cortical cells (Figure 4.7B, i).

Taken together, our data suggest that the reduction of *Verticillium* stem stripe disease symptoms under drought is associated with attenuated repression of genes involved in cell wall modification, along with the induction of genes required for cell wall reinforcement. This likely contributes to strengthening of hypocotyl cell walls, thereby limiting fungal colonization within the tissue.

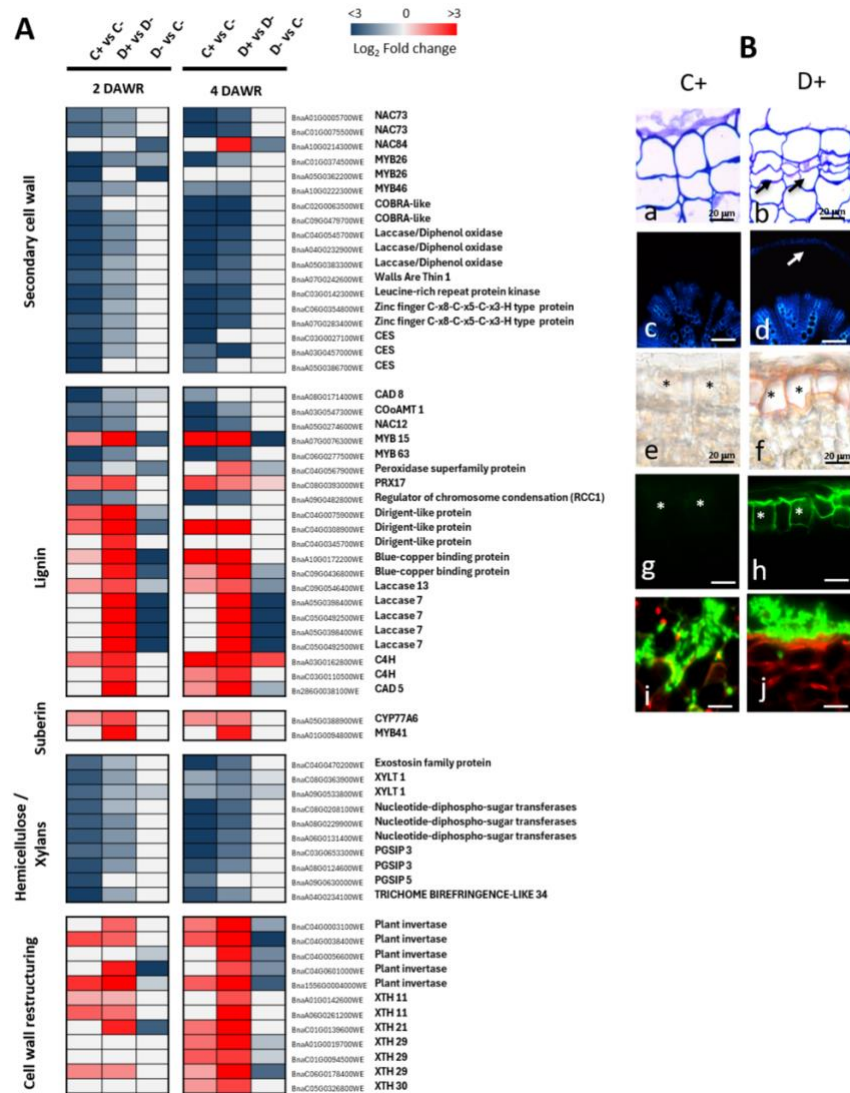


Figure 4.7 A. Heat map of significant DEG (FDR < 0.05) belonging to the GO categories related to secondary cell wall and cell wall components. The map compares the expression of exemplary genes among the different treatments (C+ vs. C-, D+ vs. D-, and D- vs. C-) at 2 and 4 Days after water regime (DAWR). B. Relative to C+ (a, c, e, and g), hypocotyls of D+ plants exhibited precocious formation of a peridermal layer (arrows in b) staining positively with berberine hemisulfate detecting lignified walls (arrow in d), Phloroglucinol-HCl staining lignin (asterisks in f) and Fluorol Yellow 088 staining suberin (h). The lignified and suberized peridermal layer of D+ hypocotyl acted as a barrier restricting the penetration of the pathogen in the inner layer (j), which was often noted in C+ hypocotyls (i). Scale bars = 25 μ m (a, b, e, - j) and 110 μ m (c-d).

4.5.6 Water regimes elicit diverse hormone responses during *V. longisporum* infection

GO term enrichment analyses revealed *Verticillium*-inoculated hypocotyls of well-watered plants to be enriched in genes pertaining to JA and gibberellin (GA) at the late infection stages (C+ vs. C- in Figure 4B), while *Verticillium*-inoculated hypocotyls of drought plants were preferentially enriched in genes related to ET at the early infection stage (D+ vs. D- in Figure 3B). To further investigate this different response, we mined the DEG within these terms (Figure 4.8). At the late infection stage (4 DAWR) *V. longisporum* infection under control water conditions (C+ vs. C-) consistently induced the expression of several genes associated with JA signalling; they included members of the *TYFY*, *WRKY* and *JAZ* families, as well as mediators of JA responses such as *ERF2*, *CULLIN 1* and two *HYDROLASES*. A similar induction was also noted for components of gibberellin signalling including *PROTEIN PHOSPHATASE 2C (PP2C)*, *PHYTOCHROME INTERACTING FACTOR 2-5 (PIF 3-5)*, *MYB 101*, and several DEG encoding glycosyl hydrolases and LRP-related proteins.

The transcript abundance of many DEG encoding ET-related genes increased in infected hypocotyls of drought stressed plants (D+ vs. D-) at 2 DAWR, with a more consistent up-regulation occurring at 4 DAWR. They included *COOPERATIVELY REGULATED BY ETHYLENE AND JASMONATE (CEF1)*, several *ERF* (1, 2, 8 and 104) and integrase-types DNA binding proteins, as well as the ET biosynthetic gene *1-AMINOCYCLO-1-CARBOXYLATE OXIDASE 4 (ACO4)*. The expression of all these genes, with the exclusion of some encoding integrase-types DNA binding proteins was also induced by the fungus at 4 DAWR in hypocotyls of well-watered plants (C+ vs. C-).

These findings suggest that diversification of hormonal responses is evoked by water regimes during infection, and that the early (2 DAWR) up-regulation of many ET related genes in hypocotyls of drought stressed plants might contribute to the delay of disease progression.

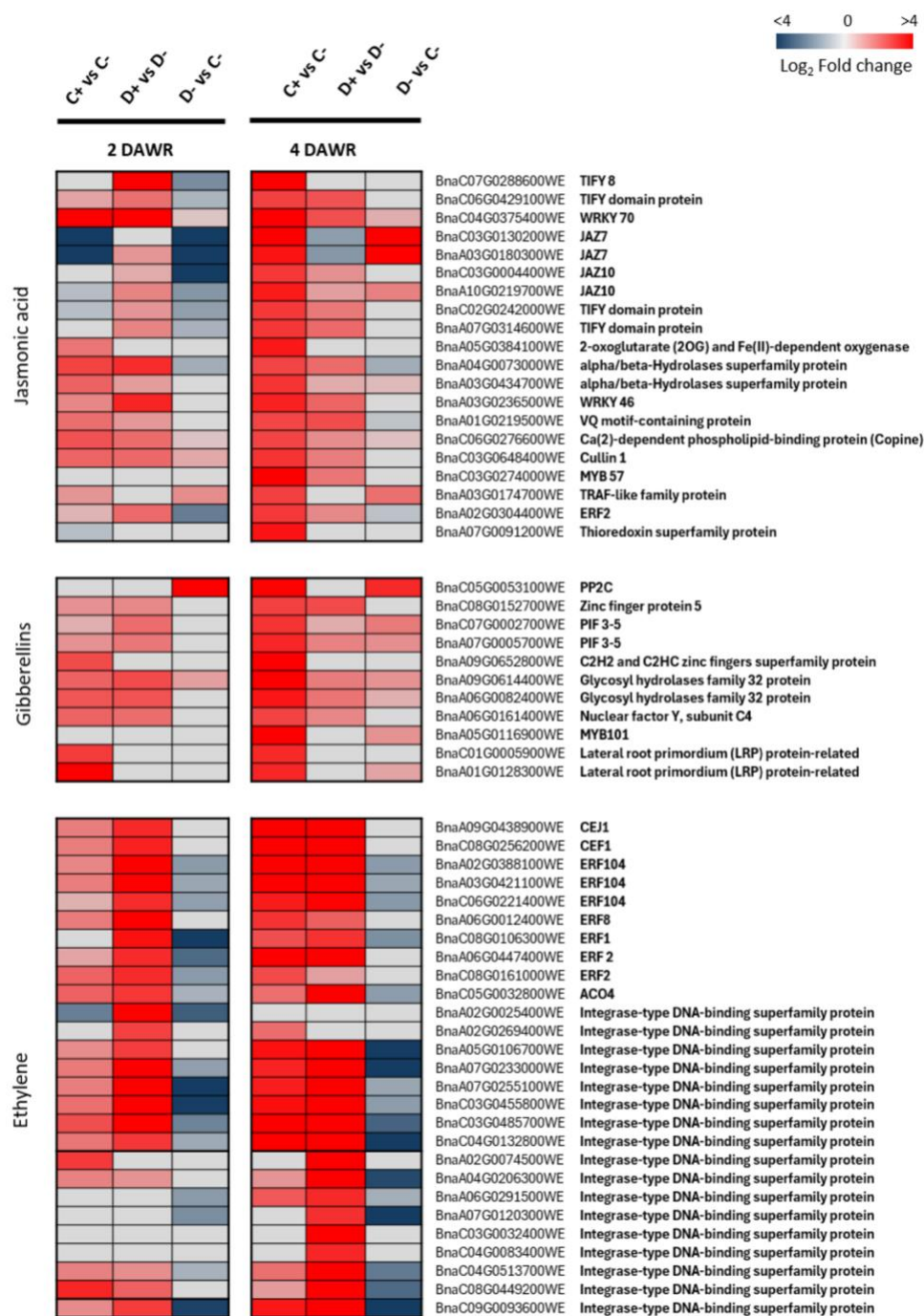


Figure 4.8 Heat map of significant DEG (FDR < 0.05) belonging to the GO categories related to hormone synthesis and signalling. The map compares the expression of exemplary genes among the different treatments (C+ vs. C-, D+ vs. D-, and D- vs. C-) at 2 and 4 Days after water regime (DAWR).

4.5.7 Co-expression and gene regulatory network analysis of differentially expressed genes

To investigate coordinated transcriptional responses underlying the treatments, WGCNA was performed using DEGs. In total, 74 co-expression modules were identified (Supplemental Figure S4.2).

Among these, the navajowhite module showed the strongest association with treatment conditions and was selected for further analysis. This module was positively correlated with drought-inoculated plants at 2 DAWR (D+, $r = 0.61$) and negatively correlated with well-watered inoculated plants at 4 DAWR (C+, $r = -0.63$, $p = 0.001$), indicating contrasting early and late transcriptional responses. The module contained 106 genes connected through 5,565 edges, forming a dense co-expression network (Supplemental Figure S4.3). Functional annotation revealed enrichment for defence, hormone signalling, and stress-responsive genes, including DOF and NAC transcription factors, ERF3, SnRK2.2, leucine-rich repeat receptor kinases, and thaumatin-like proteins. These results suggest coordinated activation of defence and hormone-regulated pathways during early infection under drought.

To further resolve regulatory relationships underlying these transcriptional patterns, a gene regulatory network (GRN) was constructed to predict transcription factor (TF)-target interactions, providing insight into the hierarchical organization of gene regulation captured within the transcriptomic dataset (Figure 4.9). The GRN comprised 1,871 genes connected by 2,247 edges. Functional categorisation of network components revealed major biological groups, including defence, cell wall-associated processes, detoxification, glucosinolate metabolism, and hormone signalling, indicating coordinated regulation of immune, structural, and metabolic pathways (Figure 4.9).

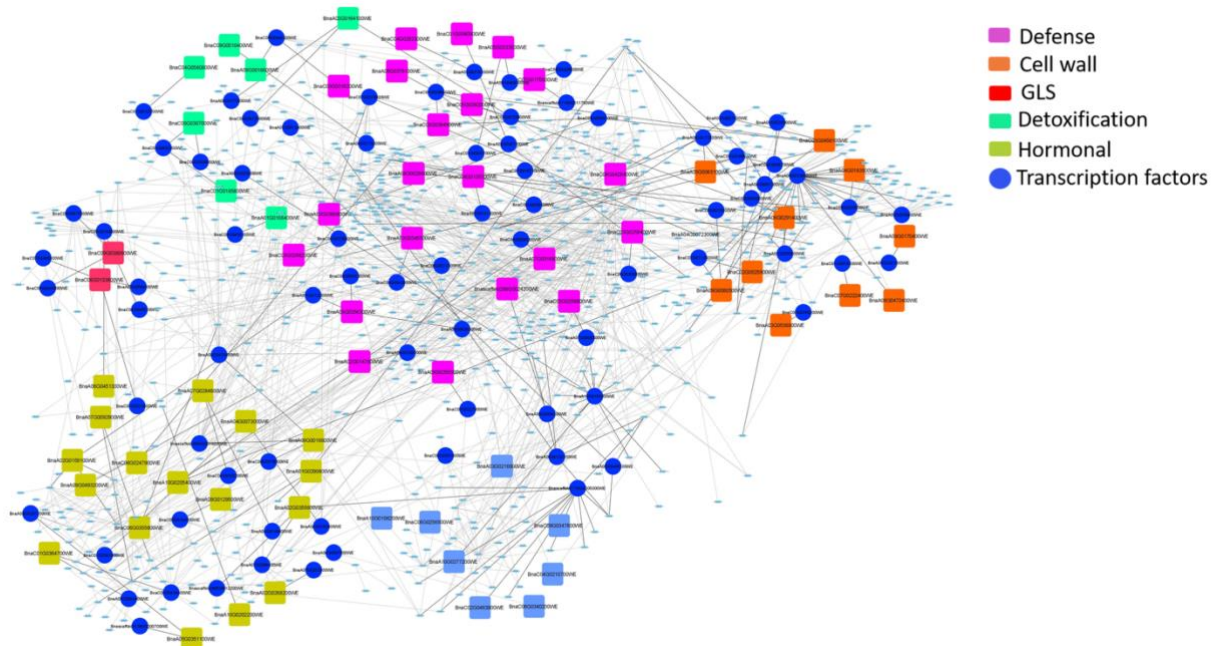
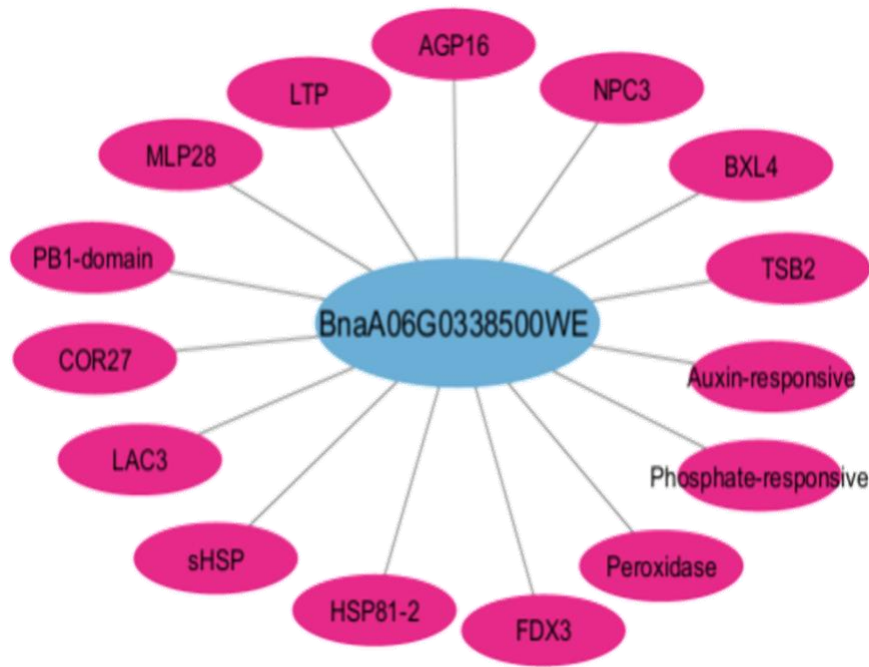


Figure 4.9 Gene regulatory network constructed from differentially expressed genes.

Network representation of DEGs showing predicted regulatory relationships between genes. Nodes represent genes and edges indicate regulatory or interaction connections. Genes are coloured according to their functional classification, including defence-related genes, cell wall-associated genes, glucosinolate (GLS) pathway components, detoxification-related genes, hormone-associated genes, light-response genes, and transcription factors.

TF-target interactions further highlighted distinct regulatory control across key pathways. Defence-related genes such as chitinases were associated with TF families including *zfp-Dof*, *NAM*, *WRKY*, and *SAM_LFY*, consistent with pathogen-responsive transcriptional regulation. Hormone signalling components were also integrated within the network, linking stress perception to downstream defence responses. In parallel, cell wall-associated genes, including *CES*, *LACCASES*, *β -1,3-GLUCANASES*, and xyloglucan-modifying enzymes, were connected to multiple TF families, indicating dynamic regulation of structural defence and cell wall remodelling. Integration of stress pathways was also evident through associations among oxidative stress genes such as *CATALASE 1*, hormone responsive regulators including *AUX/IAA* and *AP2 TFs*, and defence-related secondary metabolism genes such as *CYTOCHROME P450s* and *PROTEASE INHIBITORS*.

Notably, the bHLH transcription factor BnaA06G0338500WE represented a major hub node within the network (Figure 4.10), with 15 predicted target genes associated with hormone signalling, cell wall-related processes, and defence responses, suggesting a potential coordinating role in stress-responsive regulation.



Arabidopsis Ortholog	Putative Gene Name	Figure label
AT4G08950.1	Phosphate-responsive 1 family protein	Phosphate-responsive
AT3G07390.1	Auxin-responsive family protein	Auxin-responsive
AT2G30210.1	Laccase 3	LAC3
AT2G46330.1	Arabinogalactan protein 16	AGP16
AT2G01190.1	PB1 domain / OPB family protein	PB1-domain
AT5G42900.2	Cold-regulated gene 27 (COR27)	COR27
AT1G06460.1	Alpha-crystallin domain protein 32.1	sHSP
AT5G64100.1	Peroxidase superfamily protein	Peroxidase
AT5G64570.1	Beta-D-xylosidase 4	BXL4
AT5G56030.1	Heat shock protein 81-2 (HSP81-2)	HSP81-2
AT1G62510.1	Lipid-transfer / protease inhibitor family protein	LTP
AT5G38530.1	Tryptophan synthase beta type 2	TSB2
AT2G27510.1	Ferredoxin 3	FDX3
AT3G03520.1	Non-specific phospholipase C3	NPC3
AT1G70830.3	MLP-like protein 28	MLP28

Figure 4.10 Predicted regulatory subnetwork centred on the hub bHLH transcription factor BnaA06G0338500WE. The central blue node represents the bHLH transcription factor, while surrounding nodes indicate predicted target genes. Labels correspond to putative orthologous gene symbols or functional annotations.

4.5.8 Combined drought and *V. longisporum* stress induce localized and context-dependent changes in DNA methylation

To determine if the improved tolerance to *V. longisporum* by drought (Kashyap et al., 2026) was associated with epigenetic regulation events, genome-wide DNA methylation profiling was performed between D⁺ and D⁻ during early infection (2 DAWR). Analysis of cytosine methylation across CG, CHG, and CHH sequence contexts identified 467, 63, and 25 DML, respectively, between D⁺ and D⁻ conditions. In contrast, only a limited number of DMR were detected, with two DMR in CG, one in CHG, and none in CHH, indicating that methylation changes were primarily localized rather than occurring across extended genomic regions. Across all sequence contexts, methylation changes showed a consistent bias toward hypermethylation. In the CG context, 277 loci (59.3%) were hypermethylated and 190 loci (40.7%) were hypomethylated in D⁺ relative to D⁻. A similar trend was observed in CHG (41 hyper vs. 22 hypo) and CHH contexts (22 hyper vs. 3 hypo), although CHH changes were comparatively limited. Analysis of genomic distribution revealed that DML were predominantly located in intergenic regions. In the CG context, 164 loci (60.2%) were intergenic, whereas 108 loci (39.8%) overlapped gene bodies. A comparable distribution was observed in CHG and CHH contexts, with a greater proportion of DML located outside gene bodies. Methylation changes were limited in number and primarily localized, with a higher proportion of DML detected in intergenic regions.

To examine potential associations between DNA methylation and gene expression, DML-associated loci were compared with DEGs identified from transcriptomic analyses. A total of 21 DEGs overlapped with genic DML, while an additional 18 DEGs were associated with intergenic DML located within ± 2 kb of annotated genes (Supplemental Table S4.1,4.2). These loci were associated with both upregulated and downregulated DEGs. Classification of DML-associated DEGs based on methylation direction and expression patterns revealed that, in genic regions, hypermethylated loci were associated with 9 upregulated and 6 downregulated genes, whereas hypomethylated loci corresponded to 5 upregulated and 1 downregulated genes. In intergenic regions, hypermethylated loci were associated with 5 upregulated and 5 downregulated DEGs, while hypomethylated loci were linked to 2 upregulated and 6 downregulated genes. DML-associated DEGs included genes associated with defence and stress-responsive pathways, as illustrated by representative loci showing localized methylation and expression differences (Figure

4.11). These comprised genes encoding cytochrome P450 enzymes (e.g., *CYP81K1*), *WRKY* transcription factors, leucine-rich repeat protein kinases, flavin-containing monooxygenases, and RING/U-box proteins. In addition, several intergenic DML were located proximal to genes encoding regulatory components such as ABI5-binding proteins and growth-regulating factors.

Together, these results show that DNA methylation changes under combined drought and *V. longisporum* stress are primarily localized, enriched in intergenic regions, and associated with a subset of DEGs identified in transcriptomic analyses, with the distribution of DML-associated DEGs summarized in Supplemental Figure S4.4.

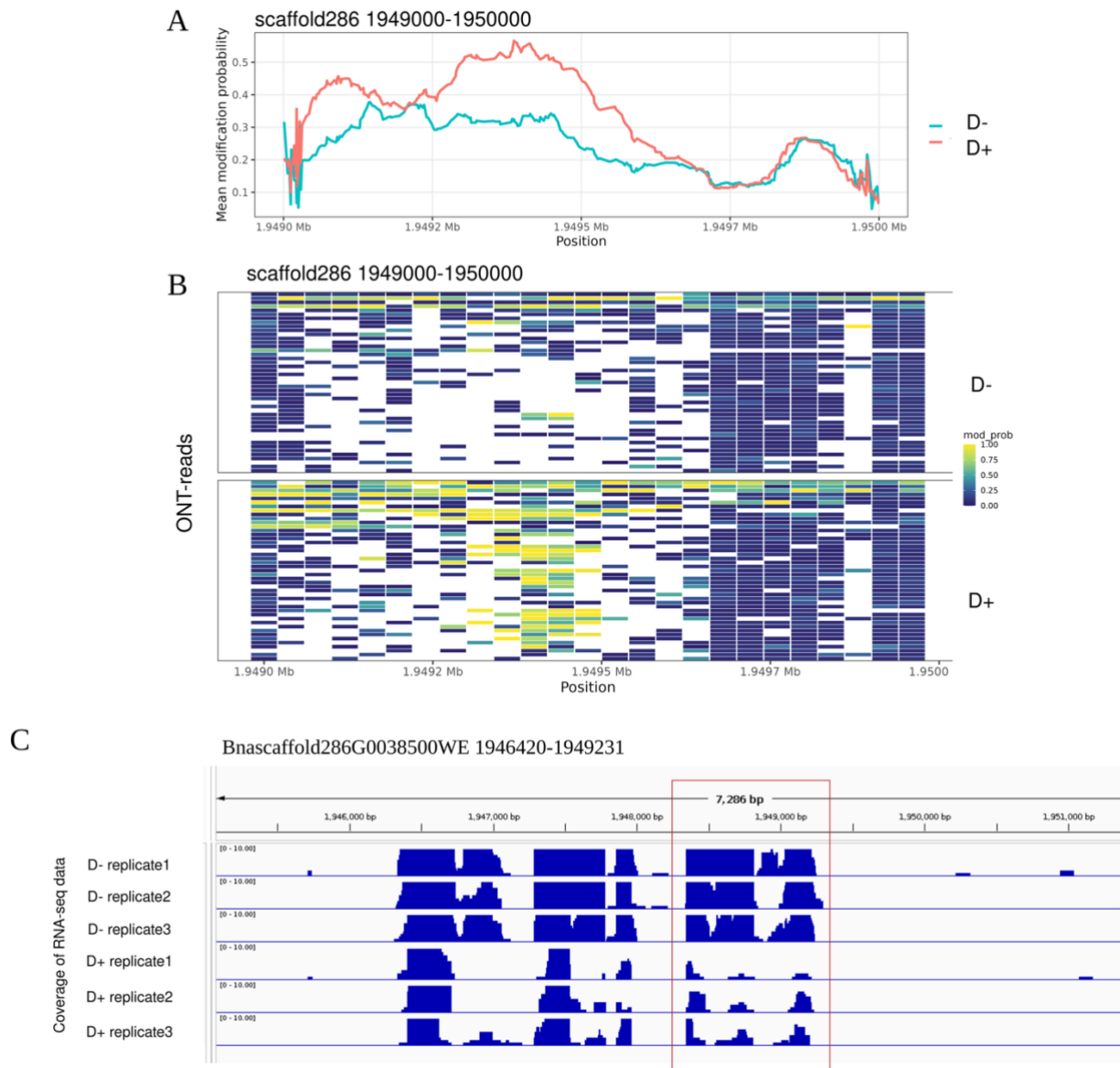


Figure 4.11 Integrated methylation and RNA-seq visualization of the scaffold286 (D-3-phosphoglycerate dehydrogenase) gene body methylation. A) Mean ONT methylation probability across scaffold286:1949000-1950000 in D⁻ and D⁺ samples. B) Read-level ONT methylation heatmap for the same region, showing per-read methylation probability in D⁻ and D⁺ groups. Colour intensity represents modification probability from low to high. C) RNA-seq coverage tracks for the associated gene region *Bnascaffold286G0038500WE* (1946420-1949231bp), comparing three biological replicates each of D⁻ and D⁺ samples. The red box highlights the methylation-affected region corresponding to panels A and B.

4.6 Discussion

4.6.1 Drought evokes global transcriptome rewiring in hypocotyls of *V. longisporum*-inoculated *B. napus* plants.

We have previously shown that drought, i.e. 30% field capacity, significantly reduced disease severity, fungal biomass, and vascular spread of *V. longisporum* in *B. napus* (Kashyap et al., 2026). Here, we characterize molecular responses associated with these effects in hypocotyl tissue, a critical transition zone for the acropetal movement of vascular pathogens.

Global transcriptome analyses revealed clear separation of samples according to water regime, infection status, and infection stage (Figure 4.1A-C), demonstrating that drought substantially altered the host transcriptional response to *V. longisporum*. Moreover, expression divergence increased from 2 to 4 DAWR, consistent with progression from early root colonization to later hypocotyl invasion (Kashyap et al., 2026). Patterns among the most strongly induced and repressed transcripts further supported these contrasting responses (Figure 4.2). In the infected comparisons (C+ vs. C– and D+ vs. D–), drought-inoculated hypocotyls (D+ vs. D–) preferentially accumulated genes associated with defence and detoxification, including *GLUTATHIONE S-TRANSFERASES*, which contribute to redox buffering and xenobiotic detoxification during stress (Dixon et al., 2010), *UDP-GLYCOSYLTRANSFERASES*, involved in secondary metabolite conjugation and defence-related metabolic homeostasis (Li et al., 2001), *CYTOCHROME P450* family members linked to specialized defence metabolism such as glucosinolate and phenylpropanoid biosynthesis (Schuler and Werck-Reichhart, 2003), and *NAC*-related transcription factors that regulate stress-responsive gene expression (Nuruzzaman et al., 2013). In contrast, well-watered inoculated plants (C+ vs. C–) showed stronger repression of genes linked to glucosinolate biosynthesis and associated metabolic pathways, including *CYP79F1*, which catalyzes aliphatic glucosinolate precursor formation (Hansen et al., 2001), *ISOPROPYLMALATE ISOMERASE 2*, associated with methionine chain elongation required for aliphatic glucosinolate production (Knill et al., 2008) and *MYB29*, a positive regulator of aliphatic glucosinolate biosynthesis (Gigolashvili et al., 2007). The drought-only comparison (D– vs. C–) revealed that water deficit in the absence of infection also modified expression of a subset of stress-responsive genes, but these changes were generally smaller than those observed in inoculated plants, indicating that the strongest transcriptional reprogramming occurred when drought and infection were combined.

GO enrichment analyses further showed that drought-inoculated plants preferentially activated defence, detoxification, and stress-signalling pathways, whereas well-watered inoculated plants displayed stronger enrichment of developmental and growth-associated categories early in infection and broader transcriptional disruption later in infection (Figures 4.3 and 4.4). Together, these data indicate that drought did not merely suppress disease indirectly, but reprogrammed host tissues toward a less permissive state for pathogen establishment.

Some of the responses were time dependent. At 2 DAWR (Figure 4.3B), drought-inoculated plants (D+ vs. D-) showed more significantly enriched defence signalling and hormone-related processes, particularly ET biosynthetic/metabolic processes and responses to molecules of fungal and bacterial origin, while detoxification related processes were less significantly enriched in D+ vs. D- at this stage. In contrast, at 4 DAWR (Figure 4.4B), detoxification and redox-related processes, including glutathione metabolism and glutathione transferase activity, were more strongly significantly enriched in D+ vs. D-. Additionally, at 4 DAWR (Figure 4.4C), cell cycle and DNA replication related processes were more significantly enriched in C+ vs. C-.

This temporal pattern is particularly relevant in the context of vascular pathogens such as *Verticillium*, where early activation of defence responses can be critical for restricting pathogen establishment. Host responses that occur after xylem invasion are often less effective, as these pathogens rapidly spread through conductive tissues and can evade localized surface defences (Fradin and Thomma, 2006; Yadeta and Thomma, 2013). Consistent with this, timely induction of host immunity and vascular occlusion responses has been associated with reduced colonization and delayed symptom development in wilt pathosystems (Dita et al., 2018). Thus, the earlier enrichment of defence-related processes in D+ vs. D- at 2 DAWR, followed by later activation of detoxification pathways, provides a mechanistic framework for the temporal regulation of host responses identified below.

4.6.2 Drought activates early defence/detoxification responses during *V. longisporum* infection

One of the most prominent features of drought-associated resistance was the preferential activation of both general and specific defence/detoxification responses from the early infection stages. Examples of genes induced in drought-inoculated hypocotyls included multiple DISEASE RESISTANCE PROTEINS of the TIR-NBS-LRR classes involved in recognition of pathogen-

derived effectors and activation of ETI (Jones and Dangl, 2006), together with several transcription factors of the *NAC*, *MYB*, and *WRKY* families (Figure 4.5A) that participate in general plant defence, secondary metabolism, and stress adaptation (Nuruzzaman et al., 2013; Pandey and Somssich, 2009). *WRKY* proteins can integrate SA and JA signalling (Rushton et al., 2010), whereas *NAC* and *MYB* factors frequently regulate both immune responses and structural defence pathways such as secondary cell wall remodelling (Nakano et al., 2015).

Drought-activation of these general defence pathways was accompanied by more specific antifungal responses, including induction of genes encoding chitinases and proteases (Figure 4.5A). Chitinases can degrade fungal cell wall chitin, whereas defence-associated proteases may inhibit pathogen enzymes or activate host immune responses (Grover, 2012; van der Hoorn and Jones, 2004). Their induction may therefore have contributed to delayed fungal invasion under drought by restricting early hyphal penetration and slowing tissue colonization, as timely expression of hydrolytic defence proteins is often associated with reduced pathogen establishment in vascular pathosystems (Fradin and Thomma, 2006; Yadeta and Thomma, 2013).

Another prominent drought-associated response involved detoxification and redox buffering. Genes related to H_2O_2 detoxification and glutathione metabolism, including several *GLUTATHIONE S-TRANSFERASES* (*GST1*, *GST3*, *GST TAU* family members) and γ -*GLUTAMYLTRANSFERASES* (*GGT1* and *GGT4*), were strongly induced, particularly under drought-inoculated conditions (Figure 4.5A). While GSTs contribute to pathogen resistance by detoxifying reactive compounds, maintaining glutathione homeostasis, and supporting redox-dependent defence signalling (Dixon et al., 2010; Gullner et al., 2018). GGT regulates extracellular glutathione turnover and recycling, thereby sustaining antioxidant capacity to alleviate ROS-induced oxidative stress (Ferretti et al., 2008). These data are supported by histochemical and biochemical analyses. Accumulation of ROS, predominantly observed in the cortex of infected hypocotyls of well-watered plants (Figure 4.5B, a, c, and d) is reduced in hypocotyls of drought plants (Figure 4.5B, b and e), which also exhibit a more intense staining for GSH in the same tissue (Figure 4.5C, b, c, and d). Induction of GSH reflects enhanced antioxidant activity (Foyer and Noctor, 2005). Furthermore, the higher level of total (GSH and SSSG) glutathione content in infected tissue under drought conditions (Figure 4.5D) implies a more active turnover between

reduced and oxidized forms to sustain ROS scavenging activity during fungal colonization (Foyer and Noctor, 2005; Noctor et al., 2012).

Drought-associated infection responses are also defined by induction of genes linked to glucosinolate metabolism, including *CYP79B3*, *CYP81F2*, *CYP81H1*, and *CYP81K1* (Figure 4.5A). *CYP79B3* participates in indole glucosinolate precursor formation, whereas *CYP81F2* is involved in synthesis of defensive indole glucosinolate derivatives that accumulate during pathogen attack (Bednarek et al., 2009; Mikkelsen et al., 2004). Glucosinolates and their hydrolysis products are important components of Brassica immunity and can inhibit fungal growth or restrict tissue colonization (Clay et al., 2009; Frerigmann et al., 2016). Interestingly, mechanisms related to SAR and cell death /hypersensitive response are preferentially induced after infection by *V. longisporum* in well-watered plants, especially during the later infection stage (Figure 4.6). While SAR, linked to SA signalling and accumulation of pathogenesis-related proteins, is a long-distance immune response, HR represents a localized defence strategy to delay pathogen progression (Mur et al., 2008; Torres et al., 2006). Activation of HR is a further indication of increased oxidative stressed caused by the pathogen under control water conditions.

Thus, water regimes evoke diverse defence mechanisms to *Verticillium*. Imposition of drought activates a plethora of both general and specific detoxification responses, some of which are related to glucosinolate synthesis and glutathione metabolism, which might contribute to delayed disease progression.

4.6.3 Cell wall remodeling during *V. longisporum* infection is altered by drought

The observation that drought mitigates the *V. longisporum* suppression of genes regulating secondary cell wall formation, hemicellulose, and xylans, while inducing key components of cell wall restructuring, lignin and suberin (Figure 4.7A) points to the role of apoplastic structural barriers in defence responses.

Verticillium longisporum, which accesses the xylem by undermining endodermal development and function (Fröschel et al., 2021), weakens structural barriers under well-watered conditions. This is reflected in the downregulation of secondary cell wall and matrix polysaccharide genes, including *CES*, *COBRA-like*, wall-associated kinase, *XYLT1*, *PGSIP3*, and *PGSIP5*, implicated in cellulose-hemicellulose organization (Scheller and Ulvskov, 2010; Somerville et al., 2004). Under drought, however, this repression is alleviated, suggesting that the host might preserve cell wall

integrity and structure. Drought also shifts the response from structural loss toward active wall remodeling. The induction of plant invertases likely increases hexose availability to fuel defence metabolism (Roitsch and González, 2004), while increased expression of, *XTH11*, *XTH21*, *XTH29*, and *XTH30* suggests controlled restructuring of xyloglucan networks (Cosgrove, 2005). The role of wall reinforcement as a defence against *V. longisporum* under drought is most evident by the attenuated suppression, or in some cases induction, of several genes involved in lignin synthesis and deposition. These include members of the *CINNAMYL ALCOHOL DEHYDROGENASE* (CAD) family, *CAFFEYL-CoA O-METHYLTRANSFERASE 1* (CCoAOMT1), and *CINNAMATE-4-HYDROXYLASE* (C4H). While CAD catalyzes the final step in monolignol biosynthesis by converting cinnamyl aldehydes into their corresponding alcohols, thus providing the direct precursors for lignin polymer formation (Barros et al., 2015; Vanholme et al., 2010), CCoAOMT1 participates in methylation reactions within the phenylpropanoid pathway, contributing to the formation of guaiacyl and syringyl lignin units that influence lignin composition and structural properties (Vanholme et al., 2010). CINNAMATE-4-HYDROXYLASE (C4H), an early cytochrome P450 enzyme in the pathway, catalyzes the hydroxylation of cinnamate to p-coumarate, thereby controlling flux into the phenylpropanoid pathway and ultimately regulating lignin biosynthesis (Boerjan et al., 2003; Vanholme et al., 2010). Oxidative lignin polymerization is catalyzed by both *LACCASES* and *PEROXIDASES*, (Barros et al., 2015; Zhao et al., 2013), some of which are induced by drought during infection.

A similar transcriptional induction under drought was also observed for two key suberin-related genes, *CYP77A6* and *MYB41*. *CYP77A6*, a cytochrome P450 monooxygenase, participates in the oxidative modification of fatty acid precursors required for the formation of aliphatic suberin components (Lashbrooke et al., 2016; Vishwanath et al., 2015), while *MYB41*, induced in infected tissue under drought but not under control water conditions, activates suberin biosynthetic pathways promoting stress-induced suberin deposition in plant tissues (Kosma et al., 2014). The drought-activation of cell wall reinforcement components in infected tissue was also confirmed histologically. Relative to well-watered plants, water stress induced the early formation of a lignified and suberized peridermal layer acting as a physical barrier to limit the spread of the fungal biomass in inner hypocotyl layers (Figure 4.7B). These anatomical changes are consistent with our previous studies (Kashyap et al., 2026). Together, these findings suggests that drought might

mitigate the weakening of cell wall structures by *V. longisporum* and induce formation of apoplastic barrier to limit the colonization of the pathogen.

4.6.4 Water regimes influence hormone responses during *V. longisporum* infection

Hormone-associated transcriptional changes provide a mechanistic bridge between fungal perception and host's response. In plant immunity, JA and ET typically act together to coordinate defence against necrotrophic and vascular pathogens, whereas GA promote growth and can antagonize defence signalling (Navarro et al., 2008; Pieterse et al., 2012). The balance and timing of these pathways are therefore critical in determining whether plants prioritize growth or defence under stress (Pieterse et al., 2012). This balance is altered by the level of water, with a shift towards JA- and GA-associated signalling responses in well-watered inoculated tissue (C+ vs. C- in Figure 4.8). This shift, most apparent during late infection (4 DAWR), is characterized by the induction of several JA-associated regulators, including *JAZ7*, *JAZ10*, and *TIFY*-domain proteins, together with transcription factors such as *WRKY70* and *WRKY46*. This pattern reflects prolonged JA pathway turnover that might indicate continued pathogen challenge rather than effective early containment (Chini et al., 2007; Thines et al., 2007). At the same time, the increased activation of GA-associated genes such as *PIF3/5*, *MYB101*, and *PP2C* suggests continued investment in growth-related processes (Navarro et al., 2008), perhaps to compensate for the suppression of cell division and proliferation processes evoked by the pathogen (Supplemental Figure S4.5). Because GA signalling can antagonize defence through DELLA-dependent mechanisms, this late JA/GA-dominated profile likely delays full activation of effective immune responses, creating a permissive window for pathogen establishment (Fradin and Thomma, 2006; Navarro et al., 2008).

In contrast to well-watered plants, the hormonal balance of drought-inoculated plants is shifted towards an early (2 DAWR) ET response. Key components of ET biosynthesis and signalling, including (*1-aminocyclopropane-1-carboxylate oxidase 4*) *ACO4* (Houben and Van de Poel, 2019) and multiple *ETHYLENE RESPONSE FACTORS* (*ERF1*, *ERF2*, *ERF8*, *ERF104*) known to bridge ET perception to response (Bethke et al., 2009; Huang et al., 2016; Lorenzo et al., 2003), are strongly induced as early as 2 DAWR under drought, whereas their broader activation under well-watered conditions mainly occurs at a later infection stage (4 DAWR). This temporal shift towards a more advanced stage of infection might be detrimental for host defence. It is well established that ERFs act as central regulators of downstream defence outputs; their early induction likely

enables coordinated activation of detoxification pathways and cell wall-associated defences counteracting the initial fungal infection phases. Besides regulating ROS homeostasis and antioxidant responses to limit oxidative stress (Apel and Hirt, 2004; Noctor et al., 2014), ERFs activate genes required for lignification, suberization, and polysaccharide restructuring (Miedes et al., 2014; Pré et al., 2008; Solano et al., 1998; Vanholme et al., 2010).

Taken together, these patterns indicate that drought reprograms hormonal signalling from a late JA/GA-dominated response toward an early ET-driven defence strategy. This shift might enable faster activation of detoxification pathways, tighter control of oxidative stress, and coordinated cell wall reinforcement, ultimately enhancing containment of *Verticillium*.

4.6.5 Co-expression and regulatory networks support integrated control of drought-associated resistance

Network analyses supported the coordinated nature of the responses identified above. Weighted gene co-expression analysis revealed the navajowhite module to be positively associated with drought-inoculated plants at 2 DAWR and negatively associated with well-watered inoculated plants at 4 DAWR (Supplemental Figure S4.3) indicating that transcriptional programs activated early under drought were reduced or delayed under well-watered conditions. Such early coordinated responses are often important for effective stress adaptation (Lämke & Bäurle 2017). Genes within this module included *NAC* and *DOF* transcription factors, *ERF3*, *SnRK2.2*, receptor-like kinases, thaumatin-like proteins, terpene synthases, and protease-related genes, linking hormone signalling with defence activation. These components align with the DEG patterns described in Figures 4.5 and 4.8, where defence, detoxification, and hormone-responsive pathways were enhanced under drought. NAC, ERF, and SnRK2 proteins are well-established regulators of drought and pathogen responses (Fujita et al., 2013; Pieterse et al., 2012). The broader gene regulatory network further connected genes involved in defence, detoxification, glucosinolate metabolism, hormone signalling, and cell wall processes (Figure 4.9) reinforcing conclusions from the DEG analyses that drought-enhanced resistance involved integrated rather than isolated pathways.

Notably, the bHLH transcription factor BnaA06G0338500WE emerged as a central hub in the regulatory network, showing predicted associations with multiple targets involved in hormone signalling, cell wall remodeling, and defence responses (Figure 4.10). Members of the bHLH

transcription factor family are increasingly recognised as important regulators integrating JA, ABA, and ROS signalling during stress adaptation (Goossens et al., 2017). Consistent with this broader functional role, another bHLH transcription factor in *B. napus*, BnaFBH3-C06, has been reported to participate in ABA-dependent osmotic stress responses (Liu et al., 2023). Although *BnaA06G0338500WE* has not yet been functionally characterized, its central position within the predicted regulatory network suggests that it may coordinate multiple protective pathways contributing to drought-enhanced resistance.

4.6.6 Localized DNA methylation changes align with early transcriptomic responses

DNA methylation profiling at 2 DAWR provided an additional layer associated with early drought-*V. longisporum* responses. Because this time point precedes extensive hypocotyl colonization (Kashyap et al., 2026), methylation changes detected at this stage are more likely to be associated with early regulatory events rather than downstream consequences of advanced infection. Consistent with this interpretation, combined drought and *V. longisporum* stress produced a limited number of DML and very few DMR, indicating that methylation changes were localized rather than genome wide. Such locus-specific methylation dynamics are consistent with a potential role in modulating gene expression under stress conditions (Law & Jacobsen, 2010; Zhang et al., 2018). A prominent feature of the methylation dataset was a bias toward hypermethylation, particularly in the CG context, together with a greater proportion of DML in intergenic regions. This pattern suggests that methylation changes may occur preferentially near regulatory regions influencing gene expression (Zhang et al., 2018). This interpretation fits well with the transcriptomic data, where drought altered the timing and specificity of defence and detoxification responses (Figure 4.3B; Figure 4.5A).

Integration of methylation and expression datasets identified a focused but functionally relevant subset of DML-associated DEGs. These included genes related to transcriptional regulation, detoxification, signalling, and cell wall modification. For example, *WRKY74*, a member of the *WRKY* family involved in plant defence and stress signalling (Eulgem and Somssich, 2007), and *CYP81K1*, a cytochrome P450 family member associated with secondary metabolism and detoxification (Bak et al., 2011), were associated with genic DML. Other DML-associated genes, including pectin lyase-like proteins, LRR kinases, RING/U-box proteins, and ABI5-binding proteins, further connect methylation changes with pathways implicated in cell wall remodeling,

pathogen perception, ubiquitin-mediated regulation, and hormone related stress signalling (Finkelstein and Lynch, 2000; Stone et al., 2005; Sun et al., 2013; Tang et al., 2017). The representative locus shown in Figure 4.11 illustrates this relationship at higher resolution, where localized hypermethylation under D⁺ is associated with differences in transcriptional activity of a detoxification-related gene. This example supports a context-dependent relationship between methylation and expression, rather than a universal pattern across all DML-associated genes. Such context dependence is expected, as the effect of DNA methylation on transcription depends on genomic location, methylation context, and local chromatin environment (Law & Jacobsen, 2010; Zhang et al., 2018). Overall, the relatively small number of DML-associated DEG supports the interpretation that methylation changes are targeted rather than widespread. DNA methylation is therefore associated with selective transcriptional responses rather than acting as a broad driver of genome-wide expression changes.

4.7 Conclusion

This study provides a robust molecular framework for understanding how drought limits *V. longisporum* colonization in *B. napus* through coordinated and time-dependent host responses. By integrating transcriptomic and epigenetic analyses, we show that drought is associated with early transcriptional reprogramming leading to activation of defence and detoxification pathways accompanied by changes in cell wall restructuring and formation of apoplastic barriers limiting the spread of the pathogen. Such changes are likely regulated by variations in hormone signalling defined by ET responses at the onset of infection. These variations, summarized in a model (Figure 4.12), are accompanied by localized and context-dependent DNA methylation changes which are associated with selective regulation of stress-responsive genes rather than acting as global regulators. However, the interpretation of these responses requires consideration of the biological complexity of plant-pathogen interactions. Unlike drought stress, which primarily elicits responses from the host plant, infection involves a continuous interaction between host and pathogen, both of which can influence the observed molecular patterns. Therefore, while many of the transcriptional and methylation changes identified in this study are consistent with enhanced defence and stress adaptation, some may also reflect pathogen-mediated manipulation of host regulatory pathways. Such manipulation is a common strategy used by fungal pathogens to suppress immunity or promote susceptibility during colonization (Lo Presti et al., 2015). This

possibility may be particularly relevant for *V. longisporum*, whose hemi-biotrophic lifestyle involves distinct biotrophic and necrotrophic phases that are likely associated with different host responses and regulatory states (Glazebrook, 2005). Consequently, the molecular signatures observed here are likely to represent the combined outcome of host defence, drought adaptation, and pathogen-driven modulation of host processes. Future studies integrating temporal sampling and functional analyses will help clarify these overlapping influences, distinguish plant defence responses from pathogen-induced regulatory changes, and provide greater insight into the role of *V. longisporum* during different stages of infection.

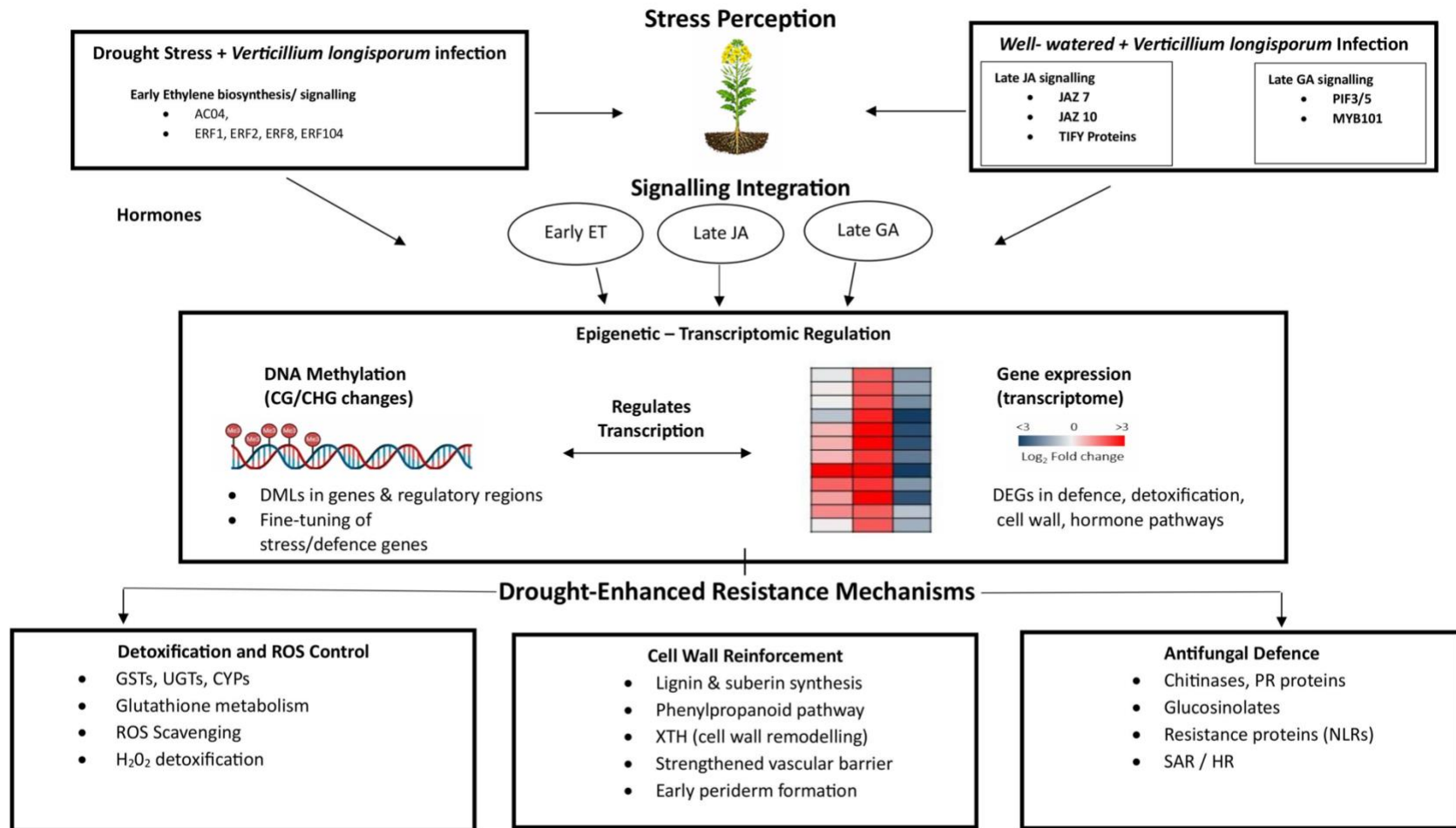


Figure 4.12 Schematic model depicting key molecular responses evoked by the combined imposition of drought and *Verticillium longisporum*. The epigenetic and transcriptomic regulation results in the activation of detoxification responses along with formation of structural barriers and production of antifungal compounds contributing to reduced fungal colonization.

5. CONCLUSION AND FUTURE RESEARCH

This study examined DNA methylation dynamics in *B. napus* during *V. longisporum* infection and under combined drought-pathogen stress using ONT sequencing to profile genome-wide methylation across CG, CHG, and CHH sequence contexts. The findings demonstrate that pathogen infection induces targeted rather than genome-wide methylation changes and that drought substantially alters host responses to *V. longisporum* at both the transcriptional and epigenetic levels.

Genome-wide methylation profiling revealed that differential methylation was concentrated primarily in CG and CHG contexts, whereas comparatively few changes were detected in the CHH context. DML were associated with genes involved in defence signalling, stress responses, and cellular regulatory processes. Furthermore, the relatively small number of DMR compared with individual DML is consistent with the possibility that DNA methylation functions as a localized regulatory mechanism that fine-tunes specific host responses during pathogen infection rather than driving broad epigenomic reprogramming. These observations are consistent with previous studies demonstrating that DNA methylation contributes to the regulation of plant defence pathways and stress-responsive processes (Downen et al., 2012; Mierziak & Wojtasik, 2024).

The integration of methylome and transcriptome analyses further exhibited that drought substantially modifies the interaction between *B. napus* and *V. longisporum*. Previous work from our laboratory showed that drought delays pathogen movement within host tissues and reduces disease severity (Kashyap et al., 2026). Consistent with these observations, combined stress was associated with extensive transcriptional reprogramming involving detoxification pathways, redox regulation, glucosinolate metabolism, hormone signalling, and cell wall modification. Enhanced lignin and suberin biosynthesis under drought conditions further suggested the establishment of structural barriers that may contribute to restricting pathogen colonization. The observation that localized methylation variation co-occurred with defence-associated transcriptional responses suggests that epigenetic regulation may contribute to the coordination of plant responses under combined stress conditions.

In addition to the biological findings, this work highlights the value of ONT sequencing for epigenomic studies in complex crop genomes. Direct methylation detection from native DNA molecules enabled high-resolution characterization of methylation patterns without the limitations associated with bisulfite conversion. Continued advances in long-read sequencing technologies, methylation phasing approaches, and methylation-calling algorithms have further improved the accuracy and applicability of long-read epigenomic analyses in polyploid crop species (Liu et al., 2021; Ni et al., 2023; Sigurpalsdottir et al., 2024).

Despite these advances, several important questions remain unresolved. Although differential methylation was associated with defence-related genes and stress-responsive pathways, the causal relationship between DNA methylation and gene expression remains unclear. Future studies should therefore focus on functional validation of candidate loci identified in this work. Emerging CRISPR/dCas9-based epigenome-editing systems provide opportunities to directly manipulate DNA methylation states without altering the underlying DNA sequence and could be used to determine whether specific methylation changes contribute to disease resistance and stress adaptation (McCutcheon et al., 2024). Such approaches may help clarify the biological significance of stress-induced methylation changes in *B. napus*. Another important area for future investigation concerns the temporal dynamics of stress-induced methylation responses. Because methylation profiles were examined at a single sampling point, it remains unclear whether the observed methylation changes represent early signalling events, downstream responses to pathogen colonization, or components of longer-term adaptation. Future studies incorporating multiple sampling points throughout pathogen infection and drought exposure will help resolve the timing and functional significance of these methylation changes (Dowen et al., 2012; Secco et al., 2015). Furthermore, the stability of these methylation marks following stress removal remains unknown and warrants further investigation. Stress-induced epigenetic modifications have been implicated in defence priming and stress memory in several plant species (Crisp et al., 2016; Lämke & Bäurle, 2017). Determining whether methylation changes associated with drought and *V. longisporum* infection persist beyond the initial stress event may provide important insights into the adaptive significance of epigenetic regulation. In addition, evaluating the potential inheritance of stress-associated methylation patterns across generations may reveal opportunities for incorporating epigenetic variation into crop improvement strategies (Kinoshita & Seki, 2014).

Finally, expanding these analyses to resistant and susceptible *B. napus* genotypes and evaluating responses under field conditions will be essential for assessing the broader biological and agricultural relevance of the methylation patterns identified in this study. Previous studies have showed substantial variation in resistance among *B. napus* cultivars (Eynck et al., 2009), highlighting the importance of determining whether the methylation signatures identified here are conserved across genotypes with contrasting levels of disease susceptibility. Such investigations will improve understanding of the relationship between epigenetic variation, stress adaptation, and disease resistance and may ultimately contribute to the development of more resilient *B. napus* cultivars.

In conclusion, this research highlights that DNA methylation is associated with plant responses to both *V. longisporum* infection and combined drought-pathogen stress and provides new insights into the epigenetic mechanisms that may contribute to stress adaptation in *B. napus*. The findings establish a foundation for future functional studies and highlight the potential of integrating epigenetic, transcriptomic, and genomic approaches to improve our understanding of plant defence regulation and support crop improvement under increasingly complex environmental conditions.

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7. APPENDICES

7.1 List of Abbreviations

BSseq:	Bisulphite sequencing
CG:	Cytosine-guanine
CHG:	Cytosine H-guanine (H = A, T, or C)
CHH:	Cytosine H-H (H = A, T, or C)
CIPK:	CBL-interacting protein kinase
DML:	Differentially methylated loci
DMR:	Differentially methylated regions
Dpi:	Days post inoculation
EDS1:	Enhanced Disease Susceptibility 1
ETI:	Effector-triggered immunity
FDR:	False discovery rate
HMW:	High molecular weight
LRR:	Leucine-rich repeat
ONT:	Oxford Nanopore Technologies
PDA:	Potato dextrose agar
PR:	Pathogenesis-related
RdDM:	RNA-directed DNA methylation
RLK:	Receptor-like kinase
ROS:	Reactive oxygen species
Rpm:	Revolutions per minute
TE:	Transposable element
UTR:	Untranslated region
DAWR:	Days after water regime imposition
DEG:	Differentially expressed gene
ET:	Ethylene
GA:	Gibberellin
GCN:	Gene co-expression network
GEO:	Gene Expression Omnibus
GO:	Gene Ontology
GRN:	Gene regulatory network
GSSG:	Oxidized glutathione
GSH:	Reduced glutathione
HR:	Hypersensitive response
JA:	Jasmonic acid
PCA:	Principal component analysis
qRT-PCR:	Quantitative real-time polymerase chain reaction
RIN:	RNA integrity number
RNA-seq:	RNA sequencing
SAR:	Systemic acquired resistance
TF:	Transcription factor

TPM:	Transcripts per million
TSS:	Transcription start site
TTS:	Transcription termination site
WGCNA:	Weighted gene co-expression network analysis

7.2 Supplementary Tables and Figures

Supplemental Table S3.1 Summary of Oxford Nanopore Technologies sequencing metrics for mock-inoculated and *Verticillium longisporum*-inoculated *Brassica napus* samples. Total bases, read counts, genome coverage, and read N50 values are shown for three biological replicates per treatment. Genome coverage was calculated based on the *Brassica napus* cv. Westar reference genome. Differences in read counts and read length distributions between treatments reflect variation in DNA fragment size distributions arising from sample-specific library preparation.

Sample ID	Treatment	Total bases (Gbp)	Coverage (×)	Read (kb)	N50	Number of reads
Mock_1	Control	30.3	26.3	10.5		3107889
Mock_2	Control	22.3	19.3	10.5		2317155
Mock_3	Control	25.5	22.2	10.5		2189449
VL_1	Infected	28.9	25.1	6		5559710
VL_2	Infected	32.1	27.9	6		6231388

VL_3	Infected	32.9	28.6	6	6360394
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Supplemental Table S3.2 Summary of differential DNA methylation across CG, CHG, and CHH sequence contexts in *Brassica napus* following *Verticillium longisporum* infection. The number of genomic windows analyzed, retained windows after coverage filtering, differentially methylated loci, and differentially methylated regions are shown for each cytosine context. DML were identified using DSS with a significance threshold of $FDR < 0.05$. Directionality of methylation change is indicated as the number and percentage of hypomethylated and hypermethylated loci in inoculated plants relative to mock-inoculated controls.

Context	Total windows	Retained windows	Retained windows (%)	Number of DML (FDR<0.05)	DMR	Hypomethylated DML	Hypomethylated (%)	Hypermethylated DML	Hypermethylated (%)
CG	960,735	798,272	83.1	824	31	516	62.6	308	37.4
CHG	961,824	912,599	94.8	267	11	197	73.8	70	26.2
CHH	962,870	942,472	97.8	19	1	10	52.6	9	47.4

Supplemental Table S4.1 List of gene-associated differentially methylated loci (genic DMLs) that overlap with differentially expressed genes in *Brassica napus* following Drought and *Verticillium longisporum* infection. The table includes gene identifiers, log₂ fold change in gene expression, methylation difference between D+ and D–, direction of methylation change (hypermethylated or hypomethylated), and predicted gene functions.

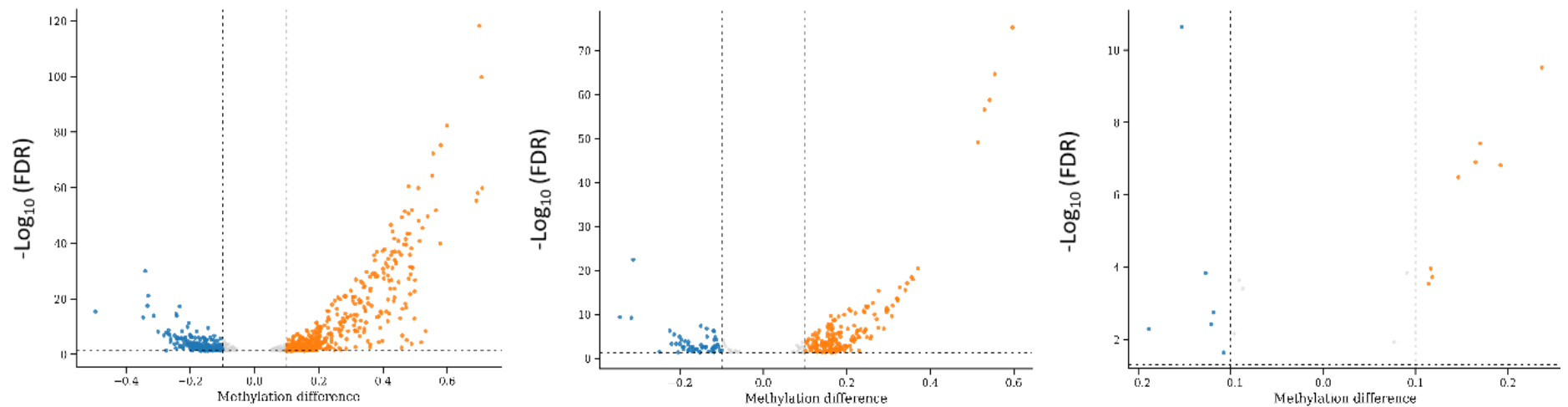
GeneID	log2 fold change	Methylation Difference (%)	Hyper/Hypo	Context	Putative Function
<i>Bnascaffold1716G0017200WE</i>	1.23993031	-0.0852307	Hyper	CG	endoplasmic reticulum-adenine nucleotide transporter 1
<i>Bnascaffold286G0038500WE</i>	-2.9539471	-0.0544725	Hyper	CG	D-3-phosphoglycerate dehydrogenase
<i>BnaA01G0036100WE</i>	-1.2905415	-0.0583094	Hyper	CG	VP1/ABI3-like 3
<i>BnaA01G0234000WE</i>	1.58644506	-0.0714145	Hyper	CG	Unknown/No Hit
<i>BnaA02G0365900WE</i>	-0.8386179	-0.0670874	Hyper	CG	WRKY DNA-binding protein 74
<i>BnaA02G0374800WE</i>	1.12610692	0.1798089	Hypo	CG	Protein kinase family protein with leucine-rich repeat domain
<i>BnaA03G0088200WE</i>	0.4669407	0.08619209	Hypo	CG	syntaxin of plants 21
<i>BnaA03G0214700WE</i>	-0.3989929	0.2272912	Hypo	CG	Protein kinase superfamily protein
<i>BnaA03G0583600WE</i>	0.84093858	-0.07628	Hyper	CG	SNF2 domain-containing protein / helicase domain-containing protein / zinc finger protein-related
<i>BnaA05G0053000WE</i>	0.39156892	-0.140782	Hyper	CG	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein

GeneID	log2 fold change	Methylation Difference (%)	Hyper/Hypo	Context	Putative Function
<i>BnaA07G0348100WE</i>	-0.5115495	-0.0800628	Hyper	CG	auxin-like 1 protein
<i>BnaC01G0424100WE</i>	-0.4558702	-0.129117	Hyper	CG	Rab escort protein
<i>BnaC04G0008800WE</i>	0.5946641	-0.1048248	Hyper	CG	ATP binding cassette subfamily B4
<i>BnaC04G0150500WE</i>	0.28427853	0.09611201	Hypo	CG	glucan synthase-like 3
<i>BnaC04G0247900WE</i>	1.01889981	-0.0784963	Hyper	CG	unknown protein; Has 24 Blast hits to 24 proteins in 8 species.
<i>BnaC06G0341900WE</i>	0.51956588	-0.0627638	Hyper	CG	PLAT/LH2 domain-containing lipoxygenase family protein
<i>BnaC07G0362800WE</i>	0.48259426	0.20389027	Hypo	CG	Protein kinase superfamily protein
<i>BnaC09G0337100WE</i>	3.68227087	0.27962145	Hypo	CG	S-locus lectin protein kinase family protein
<i>BnaC09G0523500WE</i>	2.27124977	-0.1149837	Hyper	CG	CYP81K1
<i>BnaC09G0523600WE</i>	1.01729947	-0.1376151	Hyper	CG	Telomerase activating protein Est1
<i>BnaC09G0574200WE</i>	-1.1597571	-0.0694945	Hyper	CG	Pectin lyase-like superfamily protein

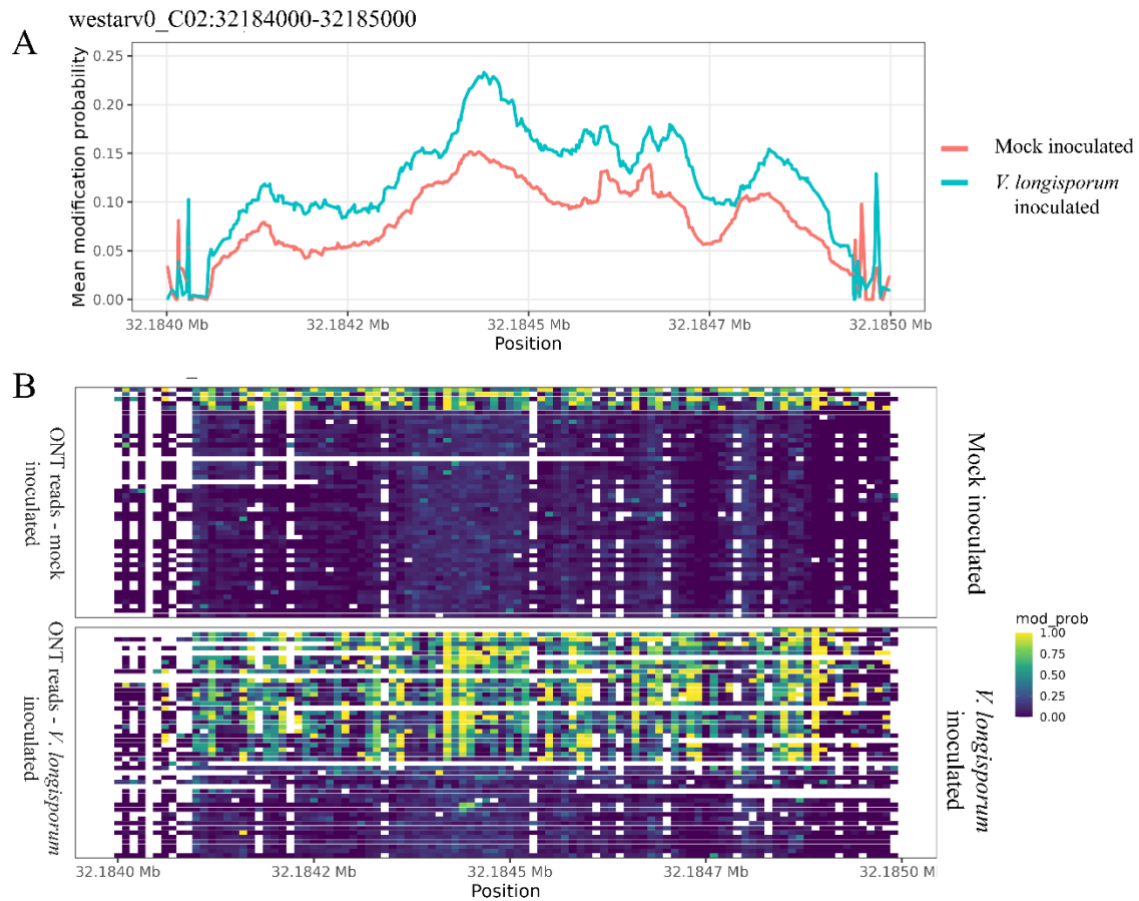
Supplemental Table S4.2. Intergenic differentially methylated loci associated with nearby differentially expressed genes in *Brassica napus* following Drought and *Verticillium longisporum* infection. The table lists DMLs located within ± 2 kb of annotated genes, including gene identifiers, log₂ fold change in gene expression, methylation difference between D+ and D–, direction of methylation change (hyper- or hypomethylation), distance of the DML from the nearest gene, sequence context (CG, CHG, or CHH), and predicted gene functions.

GeneID	Methylation difference (%)	Hyper/Hypo	Distance	Context	Putative Function
<i>BnaA02G0167200WE</i>	-0.15565	Hyper	347	CG	DNA polymerase alpha 2
<i>BnaA02G0382000WE</i>	-0.1437	Hyper	1791	CG	Lipase class 3-related protein
<i>BnaA03G0189700WE</i>	0.209395	Hypo	878	CG	Leucine-rich repeat protein kinase family protein
<i>BnaA04G0256800WE</i>	0.203308	Hypo	17	CG	DNA polymerase delta small subunit
<i>BnaA04G0276400WE</i>	-0.29307	Hyper	1751	CG	Phosphatidylinositol acetylglucosaminyltransferase
<i>BnaA07G0246500WE</i>	0.090553	Hypo	814	CG	ADC synthase superfamily protein
<i>BnaA08G0144800WE</i>	-0.09824	Hyper	1101	CHH	Flavin-binding monooxygenase family protein
<i>BnaA09G0372000WE</i>	0.163499	Hypo	1299	CG	TRAM, LAG1 and CLN8 (TLC) lipid-sensing
<i>BnaC01G0392500WE</i>	0.106178	Hypo	1845	CG	TCP-1/cpn60 chaperonin family protein
<i>BnaC03G0021100WE</i>	-0.0708	Hyper	715	CG	FUNCTIONS IN: molecular function unknown

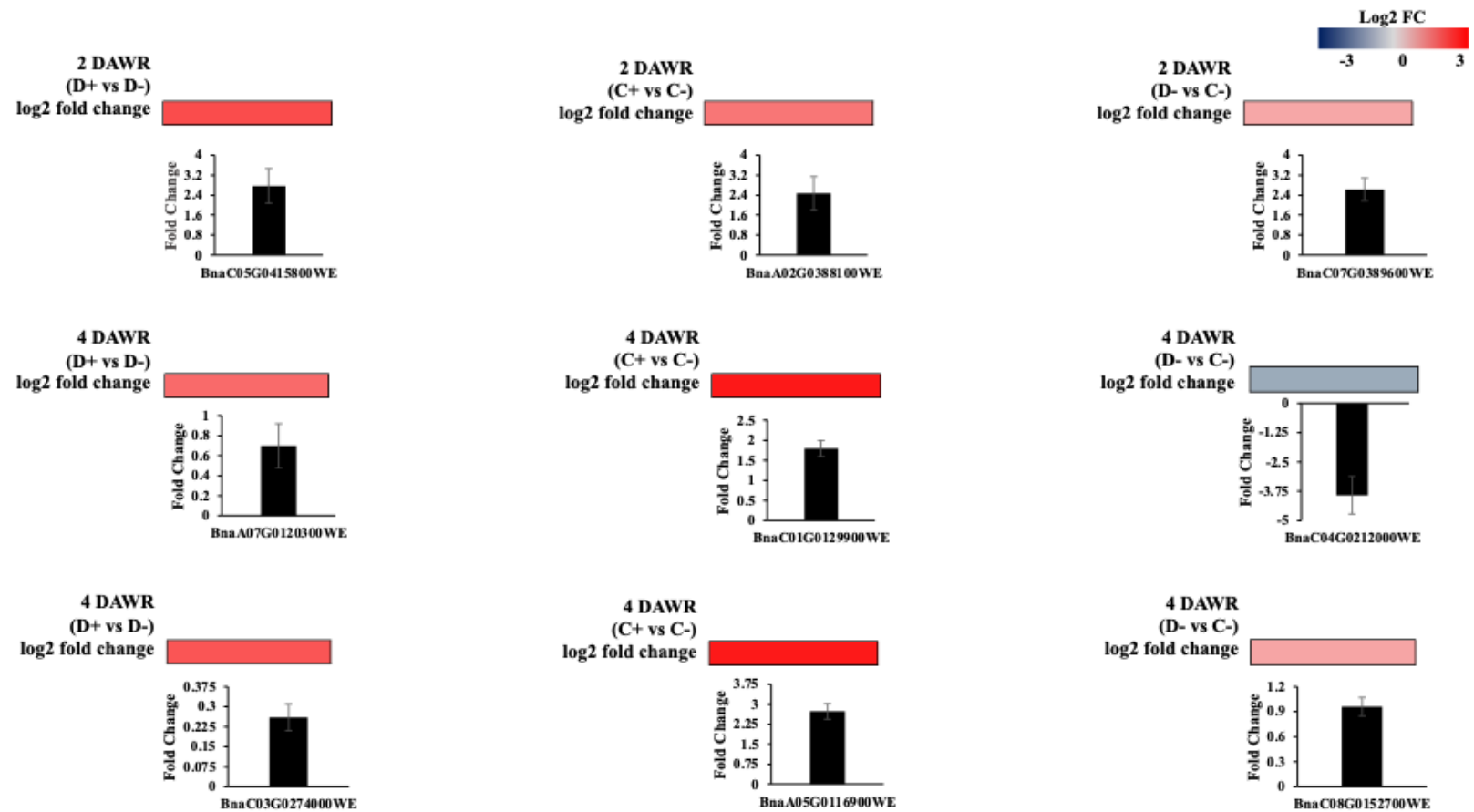
GeneID	Methylation difference (%)	Hyper/Hypo	Distance	Context	Putative Function
<i>BnaC03G0220600WE</i>	0.187703	Hypo	1880	CG	growth-regulating factor 1
<i>BnaC04G0080100WE</i>	-0.24254	Hyper	193	CHH	Sodium Bile acid symporter family
<i>BnaC04G0084100WE</i>	0.112656	Hypo	835	CG	Protein of unknown function, DUF538
<i>BnaC05G0334400WE</i>	-0.15038	Hyper	1021	CG	RING/U-box superfamily protein
<i>BnaC06G0192300WE</i>	-0.08973	Hyper	1003	CG	cationic amino acid transporter 6
<i>BnaC06G0397200WE</i>	-0.16484	Hyper	1984	CG	RNA polymerase II transcription elongation factor
<i>BnaC07G0313800WE</i>	-0.101	Hyper	96	CHG	ABI five binding protein 3
<i>BnaC08G0278500WE</i>	0.232035	Hypo	421	CG	alpha/beta-Hydrolases superfamily protein



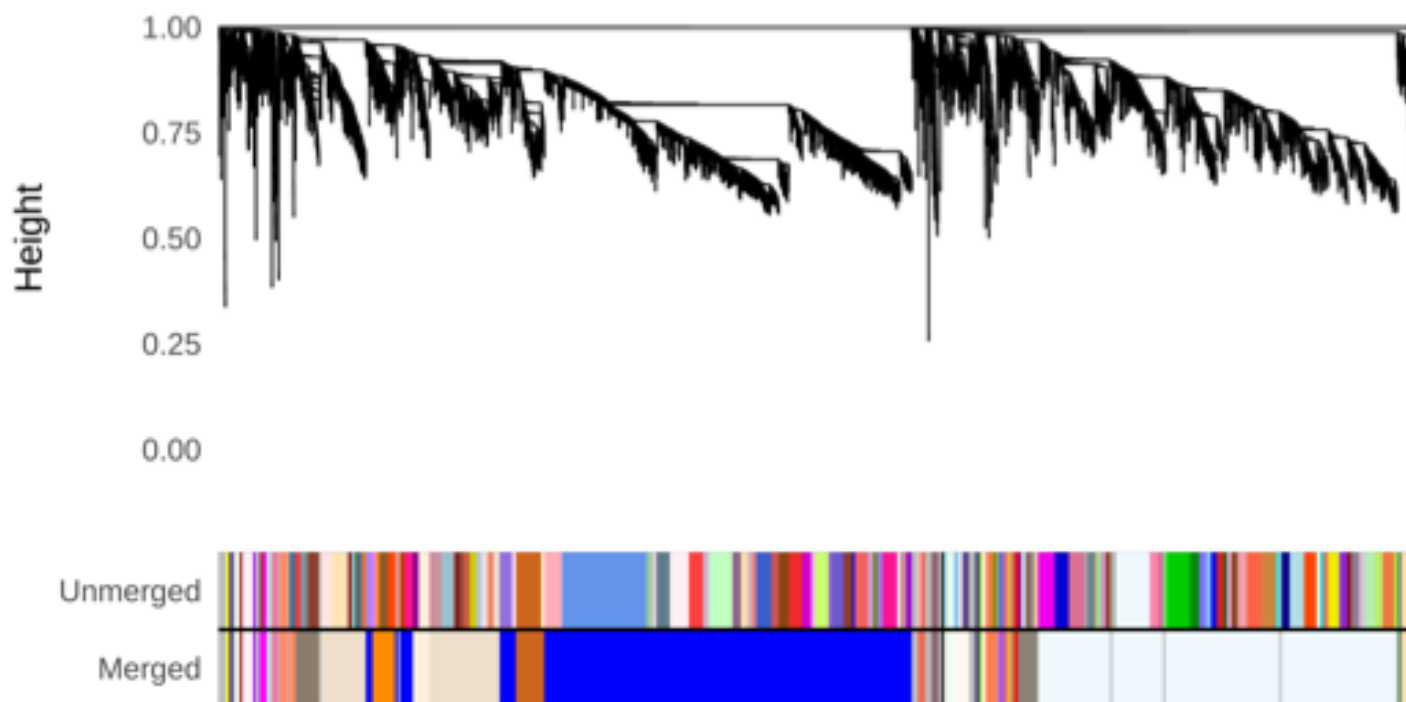
Supplemental Figure S3.1 Volcano plots of DML in response to *Verticillium longisporum* infection in *Brassica napus*. Differentially methylated loci identified between mock-inoculated and infected plants are shown for the (A) CG, (B) CHG, and (C) CHH contexts. The x-axis indicates methylation difference and the y-axis indicates $-\log_{10}(\text{FDR})$ significance level of DML. Orange and blue points denote significant hypomethylated hypermethylated and loci, respectively, while grey points represent non-significant loci.



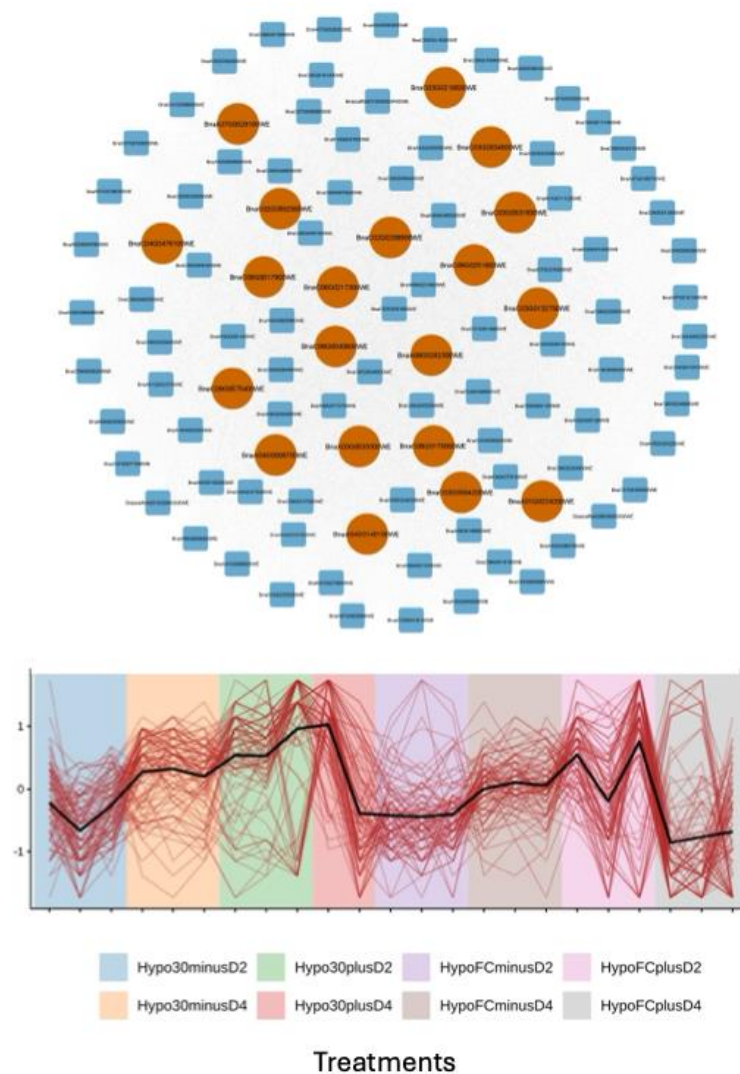
Supplemental Figure S3.2 NanoMethViz visualization of a representative gene-associated DML overlapping the defence-related gene *BnaC02G0309300WE WAKL22* (A) A representative differentially methylated locus (DML) located at chromosome C02, positions 32,184,000-32,185,000 bp overlaps *BnaC02G0309300WE*, annotated as wall-associated receptor kinase-like 22 (*WAKL22*), a gene implicated in defence responses. This DML was identified by comparing mock-inoculated and *Verticillium longisporum*-inoculated plants and exhibited a significant methylation difference ($\Delta = -0.1651$; FDR = 0.00107). The locus falls within the annotated gene interval on C02, positions 32,182,457-32,184,991 bp. A. Mean methylation modification probability across the selected region in mock- and pathogen-inoculated samples. (B) Read-level heatmaps showing methylation modification probabilities across the same region for each treatment, where each row represents an individual read and the colour scale indicates modification probability (mod_prob, 0-1).



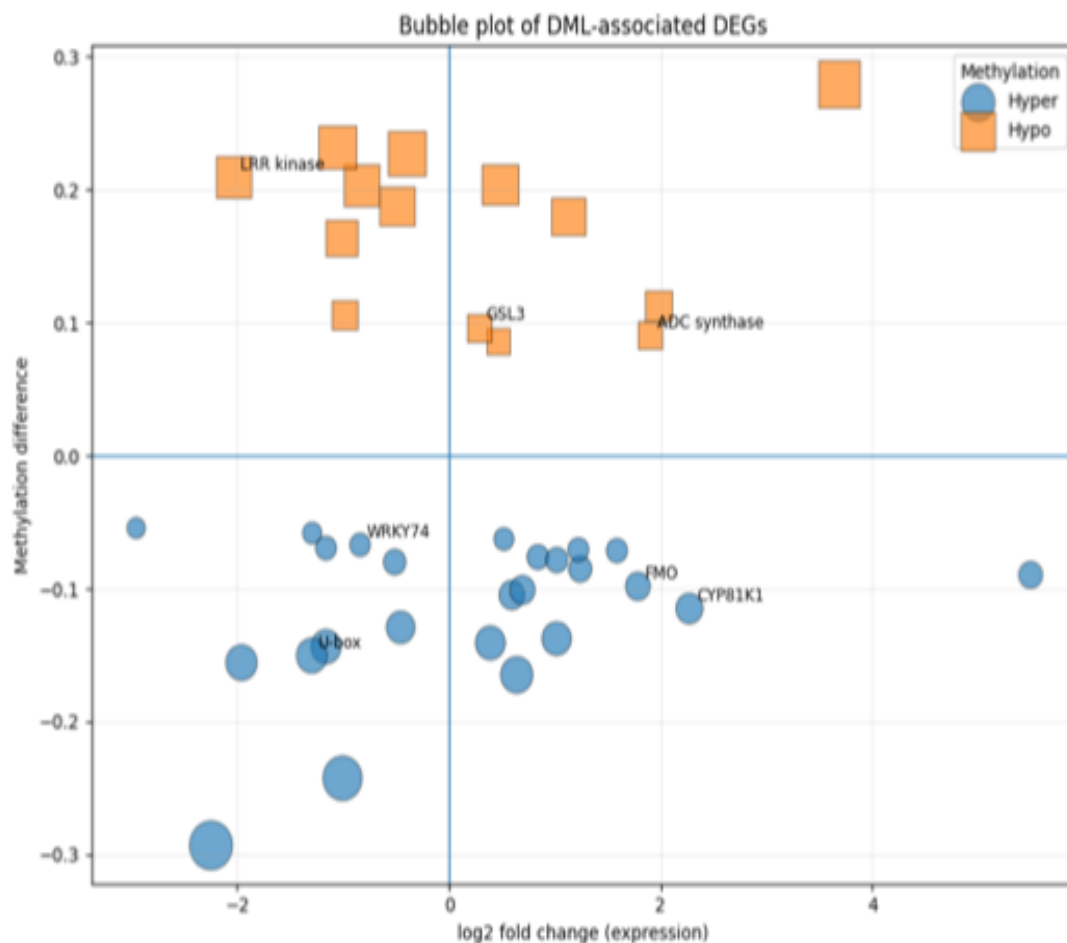
Supplemental Figure S4.1 Expression levels of nine selected genes were measured by RT-qPCR at 2 and 4 DAWR. Log₂ fold changes are shown for the comparisons C+ vs. C-, D+ vs. D-, and D- vs. C-. The selected genes represent a range of expression patterns identified in the RNA-seq dataset. Error bars indicate $\pm$ SE.



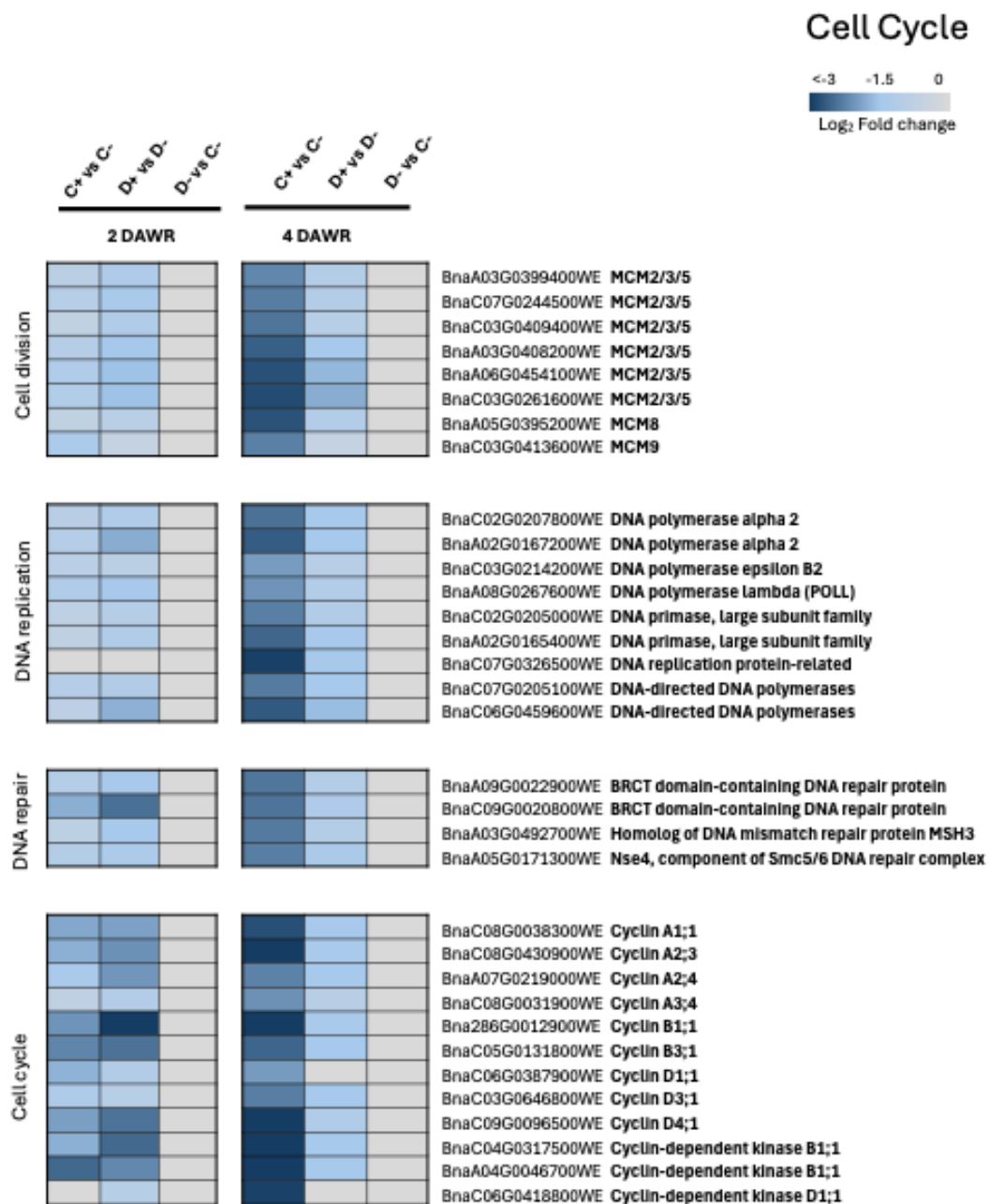
Supplemental Figure S4.2 Identification of gene co-expression modules using weighted gene co-expression network analysis (WGCNA). Hierarchical clustering dendrogram of genes based on topological overlap dissimilarity. Each branch represents genes with similar expression patterns across samples. The upper colour bar shows the modules initially identified by the dynamic tree cut algorithm (unmerged modules), while the lower colour bar represents the final set of modules after merging modules with similar eigengene expression profiles.



Supplemental Figure S4.3 Network structure and expression profile of the *navajowhite* module. Left panel: Co-expression network of genes belonging to the *navajowhite* module. Nodes represent genes and edges represent co-expression relationships based on similarity in gene expression profiles across samples. Highly connected genes are displayed as orange nodes, while other genes within the module are shown as blue nodes. Right panel: Module eigengene expression pattern of the *navajowhite* module across the experimental treatments. Individual gene expression profiles are shown as thin lines, while the bold line represents the module eigengene summarizing the overall expression trend of genes within the module across treatments.



Supplemental Figure S4.4 Relationship between DNA methylation changes and gene expression under combined drought and *Verticillium longisporum* stress. Bubble plot showing the distribution of genes associated with both differential methylation and differential expression in hypocotyl tissues at 2 days after water regime imposition (DAWR). The x-axis represents log₂ fold change in gene expression (D⁺ vs. D⁻), and the y-axis represents methylation difference (D⁺ vs. D⁻). Each bubble corresponds to a gene, with bubble size proportional to the magnitude of methylation change. Blue circles indicate hypermethylated loci, whereas orange squares indicate hypomethylated loci. Vertical and horizontal lines denote zero change in expression and methylation, respectively, dividing the plot into quadrants representing distinct combinations of transcriptional and epigenetic responses. Selected genes of interest are labeled.



Supplemental Figure S4.5 Heat map of significant DEG (FDR < 0.05) belonging to the GO categories related to regulation of cell cycle/division and DNA repairing. The map compares the expression of exemplary genes among the different treatments (C+ vs. C-, D+ vs. D-, and D- vs. C-) at 2 and 4 DAWR.