ELUCIDATION OF MECHANISMS OF HOST PLANT RESISTANCE TO WHEAT STEM SAWFLY (*Cephus cinctus* Norton) IN RELATION TO ANTIBIOSIS AND THE EARLY STEM SOLIDNESS PHENOTYPE

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Plant Science

MONTANA STATE UNIVERSITY
Bozeman, Montana

August 2023

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ABSTRACT

In the North American Great Plains, wheat stem sawfly (WSS), *Cephus cinctus* Norton is a serious pest of cultivated cereals including common bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* L. var *durum*). The solid stem phenotype is usually the basis of an effective management strategy in preventing infestation and yield loss in areas which experience pressure from large and damaging WSS populations. However solid stem expression can be negatively influenced by environmental effects and fully solid stems decrease the effectiveness of biological control of WSS by endemic parasitoids, highlighting a need for new sources of resistance outside of the solid stem phenotype. Here, we use ‘omics technologies to comprehensively examine potential mechanisms of resistance including antibiotic resistance in oat, as well as small molecules, transcripts, photosynthetic parameters and volatile organic compounds in spring and durum wheat which help to explain decreased levels of infestation and lower incidence of stem cutting observed with the early solid stem phenotype. Genes and metabolites related to cellular organization, lignin composition and stem tissue structure appear to be involved in the resistance observed in oat and are also related to the early stem solidness phenotype in spring and durum wheat. Additionally, metabolic differences in abundance of lipids and carbohydrates were observed between oat and wheat as well as in spring and durum wheat near isogenic lines. Collectively, this research provides insight into the impacts that plant metabolites and gene expression may have on plant resistance to WSS.

CHAPTER ONE

LITERATURE REVIEW

Wheat Stem Sawfly

The wheat stem sawfly (WSS), *Cephus cinctus* Norton (Hymenoptera: Cephidae), is a major pest of common bread wheat *Triticum aestivum* L. Wheat stem sawfly is indigenous to North America and was first described as a pest of wild rye, *Elymus sp.* L., wheatgrasses, *Agropyron spp.* L., and other members of the Poaceae family in the late 1800's (Ainslie, 1929). As wheat production increased in the United States and Canada and the prairie was converted to farmland, WSS adapted from its native hosts to infest cultivated grasses including spring and winter wheat, durum wheat (*Triticum durum* Desf.) and barley (*Hordeum vulgare* L.). WSS infestation has resulted in devastating yield and economic losses throughout the Great Plains of North America (Beres et al., 2007; Morrill et al., 1998). Although historically WSS has been considered to be a pest of the Great Plains, bioclimatic simulation models suggest that changing climatic conditions will allow WSS to expand their range throughout much of the southwestern United States and further to the north in western Canada (Olfert et al., 2018).

Life Cycle

WSS have one generation per year and adults emerge in late spring when climatic conditions are favorable, usually when warm weather follows a period of rain (Criddle, 1923; Wallace & McNeal, 1966). Adult emergence typically occurs from May to July depending on latitude (Ainslie, 1929). Adult WSS do not feed, and live for up to 8 days in favorable

environmental conditions (Wallace & McNeal, 1966). Adult WSS females oviposit in wheat stems shortly after emergence and are able to reproduce parthenogenically when necessary, laying viable eggs regardless of whether fertilization occurs. Females have the potential to lay up to 50 eggs, and usually lay a single egg per stem (Ainslie, 1929). However, WSS females cannot discriminate between infested and uninfested stems and will regularly oviposit in stems that already contain eggs (Buteler et al., 2009).

While stems may contain eggs from more than one female, the larvae are cannibalistic, and only one larvae per stem will survive to maturity (Wallace & McNeal, 1966). Larvae feed on parenchyma tissue lining the inside of the stem throughout the growing season, causing damage to multiple internodes. Before entering diapause, larvae prepare a hibernaculum by cutting a ring around the inside base of the stem, plugging the hole with frass, and forming a cocoon within the stem, which allows them to overwinter in the stubble even through extreme temperatures and severe weather events (Beres et al., 2007).

Yield Loss Due to WSS

Yield loss due to damage from WSS larval feeding is difficult to quantify since adult WSS females prefer the primary stems and regularly choose taller stems with larger diameters, which are more likely to be high yielding (Buteler et al., 2008; Morrill et al., 2000). Several studies have determined that the stem boring activity of the larvae damages parenchyma tissue, xylem and phloem which negatively impacts photosynthetic ability, particularly in conditions of drought or nutrient deficiency (Delaney et al., 2010; Macedo et al., 2005). This causes decreased seed weight, grain quality and number of seeds per head, lowering overall yield even when larvae do not reach maturity (Buteler et al., 2008; Delaney et al., 2010; Holmes, 1977; Morrill et

al., 1992; Morrill et al., 2000; Morrill et al., 1994; Weiss & Morrill, 1992). The stem cutting action of the mature larvae also weakens the stem which makes plants susceptible to lodging when exposed to wind, rain or gravity (Ainslie, 1920; Criddle, 1923; Morrill et al., 1998; Sherman et al., 2010). Additional yield loss occurs once stems are lodged because this makes it difficult to harvest the grain through conventional means.

Control Methods

WSS is notoriously difficult to control using chemical methods, with cultural and biological approaches being partially effective (Beres et al., 2011). Early control measures for wheat stem sawfly included cultural methods such as plowing under infested stubble, destruction of stubs by fire, early harvest and planting trap crops (Criddle, 1923; Seamans, 1928). These methods were either ineffective, inefficient or even damaging to crop production and were never widely adopted as successful means of WSS control. Swathing is often used as a management strategy to prevent yield loss due to lodging, but this requires additional equipment and labor and does not prevent yield losses from larval feeding (Beres et al., 2011; Holmes & Peterson, 1965). Crop rotation and delayed planting of some crops are sometimes effective in decreasing WSS populations from year to year, but this is not always a feasible option for producers (Beres et al., 2011; Morrill & Kushnak, 1999).

Several species of hymenopterans are known to parasitize WSS, but in wheat fields, *Bracon cephi* (Gahan) (Hymenoptera: Braconidae) and *Bracon lissogaster* Muesebeck (Hymenoptera: Braconidae) are the only parasitoids that consistently target WSS larvae. Parasitism of WSS larvae reduces yield loss due to larval feeding and stems containing parasitized larvae have greater seed weight and number of seeds per head than stems with larvae

which were not parasitized (Buteler et al., 2008). Several studies have also shown that the presence of parasitoids can decrease populations of WSS over time (Ainslie, 1929; Morrill et al., 1994; Morrill et al., 1998). Unfortunately, rates of parasitism in wheat are often not as high as in other host grasses, so control of sawfly populations by parasitoids from year to year is inconsistent (Runyon, 2001). Additionally, methods of WSS control such as swathing and the use of solid stemmed cultivars can negatively affect parasitoid populations (Rand et al., 2020; Rand et al., 2012; Runyon, 2001).

Contact insecticides generally are not effective against WSS because larvae are protected inside the stem and adult sawflies emerge over a period of weeks which makes it hard to time applications. Applications of systemic insecticides at the time of planting do not result in high enough concentrations to kill larvae by the time they hatch inside the stem. In addition, systemic insecticides are not always translocated in high concentrations to the internodes where sawfly larvae are located. From 2015 to 2019, Thimet 20-G, an organophosphate, was approved for special use in Montana to control WSS in spring and winter wheat. While effective, this insecticide is highly toxic to humans and other mammals, must be applied at least 85 days before harvest and was ultimately not adopted by producers (Wanner & Tharp, 2015).

The availability of solid stem cultivars provides producers with a valuable tool to prevent yield loss due to WSS infestation and planting of resistance solid-stemmed cultivars is currently the easiest and most popular control measure for WSS. Use of solid stem cultivars is a widely adopted and effective management strategy for WSS, and can lead to long-term reduction of sawfly population pressure (Bekkerman & Weaver, 2018). However, this strategy of utilizing host plant resistance via the solid stem phenotype does not facilitate growth of braconid

parasitoid populations as parasitism by both *B. cephi* and *B. lissogaster* is often greater in hollow stemmed wheat cultivars (Buteler et al., 2015a; Talbert et al., 2014). In combination, the trophic interactions of host plant resistance with WSS and its braconid parasitoids are most effective at reducing WSS populations over time. Exploration of stem solidness as well as additional sources of host plant resistance offers a unique opportunity to optimize this tritrophic system to maximize WSS control.

Host Plant Selection

Infestation by WSS has been recorded in many species of native and cultivated grasses, including spring, winter and durum wheats (*Triticum aestivum* L., *Triticum turgidum subsp. durum*), barley (*Hordeum vulgare* L.), oat (*Avena sativa L. and Avena fatua L.*), rye (*Secale cereale* L.), *Agropyron* spp., and downy brome (*Bromus tectorum* L.). In most host plants of WSS, females seem to prefer to lay eggs in taller plants with larger stem diameters (Ainslie, 1929; Buteler et al., 2009; Morrill et al., 2000; Wallace & McNeal, 1966). Although only one larvae per stem can survive to maturity, females do not discriminate between infested and uninfested stems and will lay eggs in stems which already contain eggs or larvae (Buteler et al., 2009).

The exact mechanism by which a female sawfly chooses a host is still unknown, although they appear to be influenced by some physiological characteristics such as plant height, diameter of the stem and stem solidness as well as volatile compounds emitted from leaves and stems (Buteler et al., 2008; Piesik et al. 2008; Morrill et al., 2000). While not much is known about how WSS or their braconid parasitoids interact with visual stimuli, most other Hymenopteran species are able to perceive wavelengths in the visible spectrum between 300-700 nm (Lebhardt

& Desplan, 2017; Peitsch et al., 1992). Female WSS exhibit a number of behaviors to examine potential host stems including walking up and down the stem, antennae and abdominal tapping and insertion of their saw-like ovipositors, bringing them in contact with both volatile organic compounds (VOCs) as well as compounds present on the plant surface (Achhami et al., 2021; Ainslie, 1929; Buteler et al., 2009; Varella et al., 2017).

Several uncharacterized odorant receptor (OR) genes are present in the WSS genome, indicating a role of olfactory perception in host seeking behaviors (Gress 2014, Robertson et al., 2018). Behavioral studies have implicated several volatile organic compounds that play a role in host plant selection by ovipositing females including Z-3-hexenyl acetate, β-ocimene and Z-3-hexen-1-ol (Piesik et al., 2008). The presence of these compounds in volatile blends have different associations with resistance to WSS depending on plant species. Females are attracted to spring wheat cultivars which have greater emissions of Z-3-hexenyl acetate, β-ocimene and Z-3-hexen-1-ol (Piesik et al., 2008; Weaver et al., 2009). More eggs are laid in cultivars of winter wheat that emit more β-ocimene and cultivars of spring wheat and barley that emit more Z-3-hexenyl acetate (Achhami et al., 2021; Buteler et al., 2010; Weaver et al., 2009). Characterization of plant traits relating to spectral reflectance and quantification of volatile compounds emitted by resistant and susceptible plants may be able to explain differences in infestation rates and influence on behavior of WSS females.

Infestation occurs regardless of host suitability, with WSS females often laying eggs in species such as downy brome or oat where larval survivorship is low or non-existent (Criddle, 1923; Perez-Mendoza et al., 2006; Sing, 2002). Cultivated oat, wild oat and barley have all been shown to exhibit some resistance to WSS due to antibiosis (Criddle, 1923; Sing, 2002; Varella et

al., 2018). In these species, female WSS choose to oviposit but larvae can die in the stem and often do not survive to become adults.

Cultivated oat and wild oat are the only host plants that exhibit complete resistance to WSS. Female WSS will choose to oviposit in oat and wild oat plants, but the larvae die in the stem before reaching maturity (Criddle, 1923; Roemhild, 1954; Sing, 2002). Though currently unknown, illuminating the mechanism for this interaction is an important step towards developing new cultivars with possible antibiotic resistance to WSS.

Genetic Resistance

A quantitative trait locus (QTL) is a region of the genome that contains a gene or suite of genes that are associated with a particular phenotypic trait. These loci are identified by conducting experiments to determine associations between phenotypes and polymorphic genetic markers which are linked to the genomic region of interest. Studies over the years have identified many QTLS for resistance to WSS in spring, winter and durum wheat but not all are associated with the solid stem phenotype (Joukhadar et al., 2013; Sherman et al., 2010; Varella et al., 2019b).

Solid Stem Trait.

In susceptible crop species, the primary defense against WSS is the development and planting of cultivars with the solid stem trait. Stems that are solid, or filled with pith, have been shown to stop or slow larval growth and maturation because larvae are not able to move easily inside the stem (Holmes & Peterson, 1962; Sherman et al., 2010). For this reason, the planting of solid stemmed cultivars often results in less damage from stem cutting, decreased survival during

overwintering and even reduced weight, size and fecundity of females the following season (Cárcamo et al., 2005). The solid stem trait is also highly heritable, making it a prime candidate for developing cultivars resistant to WSS (Cook et al., 2019).

Stem solidness has been studied extensively over the years in relation to plant resistance to WSS. The spring wheat cultivar Rescue, the first solid stemmed cultivar in North America, was developed in the 1940s using the solid stem trait from a Portuguese wheat landrace, S-615 (Kemp, 1934; Platt et al., 1948). Over the years, breeding efforts have improved the agronomic performance of solid stemmed cultivars with the S-615 background and expanded knowledge of additional genetic sources of the solid stem phenotype (Sherman et al., 2010; Varella et al., 2015; Weaver et al., 2009).

Unfortunately, solid stems do not completely inhibit WSS infestation and stem cutting as high as 30% has been observed in solid and semi solid stemmed varieties (Platt et al., 1948; Sherman et al., 2010). Higher than expected levels of stem cutting may be explained by year-to-year variation in solid stemmed varieties due to differences in location, planting density and light intensity, as well as water and nutrient availability (Beres et al., 2011; Beres et al., 2017; Delaney et al., 2010; Pluta et al., 2021; Roemhild, 1954).

3B Locus and WSS Resistance.

QTL mapping in a double haploid winter wheat population derived from a Rampart (PI 593889) (Bruckner et al., 1997)/Jerry (PI 632433) cross first showed that the solid stem trait was associated with the QTL *Qss.msub-3BL* which was found to contribute 76% of the total variation for stem solidness (Cook et al., 2004). This QTL is also responsible for variation in stem solidness in spring and durum wheat and does not appear to cause any reduction in yield

potential (Cook et al., 2004; Houshmand et al., 2007; Kalous, 2011; Nilsen, 2017). In both durum and spring wheat mapping populations, Kofa/W9262-260D3 and Lillian/Vesper respectively, the majority of stem solidness variation was explained by the *SSt1* interval on the long arm of chromosome 3B (Nilsen, 2017; Nilsen et al., 2020). This indicates that *SSt1* and the 3B QTL in spring wheat share this common genomic interval. Other minor genes controlling stem solidness are located on chromosomes 3A, 3B, 3D, 5A, 5B and 5D (Cook et al., 2019; Cook et al., 2004; Houshmand et al., 2007; International Wheat Genome Sequencing, 2018; Nilsen, 2017; Varella et al., 2019a). Minor QTL on chromosomes 2A and 4A in durum wheat and 2D and 5A in spring wheat have also been found that have a synergistic effect with *SSt1* to cause higher levels of stem solidness (Nilsen, 2017).

WSS females show preference for some Montana cultivars of spring wheat over others, with some cultivars experiencing higher levels of infestation and increased stem cutting. Three alleles have been identified that confer differing levels of stem solidness. The Rescue/Choteau (PI633974) allele (*Qss.msub-3BLb*) is associated with solid stems at all stages of development, the Conan allele (PI607549) (*Qss.msub-3BLc*) is associated with stem solidness early in plant development and the Reeder (PI613586) (Sherman et al., 2010) allele (*Qss.msub-3BLa*) is associated with a hollow stem phenotype (Cook et al., 2019; Varella, 2016). Differences in infestation and stem cutting rates are not solely defined by the solid stem trait. For example, Conan (WestBred, LLC), which loses the solid stem trait as it develops, exhibits more resistance to stem cutting than expected based on solidness alone (Sherman et al., 2010; Talbert et al., 2014).

The Spring Wheat Breeding Program at Montana State University developed near isogenic lines to study the effect of the 3B QTL on sawfly damage and other agronomic traits (Varella et al., 2016; Varella et al., 2017). These spring wheat lines are nearly identical genetically except for alleles at the 3B locus that confer varying degrees of stem solidness. In spring wheat, the early stem solidness allele from Conan, *Qss.msub-3BLc*, confers the plant with solid stems early in development, offering some degree of resistance at a critical time during the time of flight and oviposition period of WSS females. Compared to hollow stemmed lines with the *3BLa* allele, lines with the *3BLc* allele for early stem solidness experience fewer ovipositor insertions and fewer eggs laid by WSS females and lower stem cutting overall (Varella et al., 2017; Varella et al., 2016).

In recent years, promising developments have been made towards resolving the 3B QTL and identifying the gene or genes responsible for stem solidness at this locus. With the recent release of IWGSC RefSeq v1.0, a total of 160 high confidence genes at the *SSt1* locus were differentially expressed between spring wheat lines with contrasting levels of stem solidness (International Wheat Genome Sequencing, 2018). Increased copy number variation of one of these genes, a DNA-binding one-zinc finger transcription factor *TraesCS3B01G608800*, was associated with increased stem solidness (International Wheat Genome Sequencing, 2018; Nilsen, 2017). In durum wheat, increased copy number variation of *TRITD3Bv1G280530*, a putative Dof zinc finger protein (*TdDof*), was found in solid stemmed lines CDC Fortitude and W9262-260D3. Deletion of a portion of the *SSt1* region containing this gene led to loss of phenotypic expression of stem solidness, confirming that *TdDof* controls the stem solidness trait in durum wheat (Nilsen et al., 2020).

Although several studies have explored the effect of the 3B QTL on stem solidness and sawfly behavior in greenhouse and field settings, the precise mechanism responsible for the observed resistance in spring wheat is still unknown.

3A Durum Locus and WSS Resistance.

QTL analysis of a durum wheat mapping population identified a QTL associated with resistance and early stem solidness on chromosome 3A (Varella et al., 2019b). In near isogenic lines developed from the resistant cultivar Pierce (PI 632366) and the susceptible landrace SD9 (PI 41353), early stem solidness was observed in lines with the Pierce allele (*Qss.msub-3ALb*). *Qss.msub-3ALb* also caused a 25% reduction in stem cutting, while SD9 contributed the *Qss.msub-3ALa* allele associated with higher incidence of stem cutting. More stem boring was observed with the *3ALa* allele as well meaning that larval mortality does not happen as quickly as with the Pierce allele. In spring wheat, a QTL on chromosome 3A was also found to be associated with larval mortality, but it is unknown whether the same mechanism is responsible in durum wheat (Varella, 2016). Since spring wheat cultivar Conan experiences similar effects from the observed temporal changes in stem solidness, further exploration of early stem solidness in both spring wheat and durum lines can begin identifying candidate genes for these QTL and help us to better understand the role of the early stem solidness trait in sawfly resistance.

‘Omics Technologies

In recent years, technological advancements have made it possible to explore mechanisms of plant physiology, development, defense and resistance. “Omics” technologies, broadly spanning genomics, transcriptomics, proteomics, and metabolomics, have been used to

study gene expression and plant environmental response in hundreds of species. These approaches can be used to integrate information from genotype to phenotype and elucidate genes, proteins and metabolites through analysis of the transcriptome, proteome and metabolome respectively. These technologies have broad implications for the future of genome research in many crops, including tetraploid and hexaploid wheat, but these methods have not been used extensively to study Montana cultivars.

Transcriptomics, or analysis of the gene transcripts in a system, can offer a picture of which genes may be involved in plant response to abiotic and biotic stresses (Dhokane et al., 2016; Y. Li et al., 2021; Liu et al., 2012; Yue et al., 2022). Gene transcripts are tissue-specific and offer a way to pinpoint differences in gene expression that can provide information about a wide range of cellular processes (Shi et al., 2021). Transcriptomics analysis of spring wheat cultivars infested with WSS have identified the involvement of the phenylpropanoid and phosphate pentose pathways in plant defense against larval feeding, but studies have not been performed to identify sources of inherent resistance (Biyiklioglu et al., 2018). Transcriptomics can also be a valuable tool in identifying candidate genes when combined with QTL analysis (Jian et al., 2019; X. Wang et al., 2020).

Metabolite analysis or metabolomics can also be used to obtain a more complete picture of the physiological differences between resistant and susceptible plants. These small molecules are involved in every aspect of plant function and change rapidly in response to environmental changes, making them a perfect target to obtain a “snapshot” of the state of the plant at a specific point in time. In some cases, metabolite profiles are heritable and metabolomics can be used to identify and characterize QTLs associated with a wide range of plant traits (Carreno-Quintero et

al., 2012; Li et al., 2019; Nunes-Nesi et al., 2019). WSS infestation induces changes in the metabolome of several spring and durum wheat cultivars, particularly in the alkaloid, benzenoid, carbohydrate and lipid chemical classes (Biyiklioglu et al., 2018; Lavergne et al., 2020; Nilsen et al., 2020). Together, QTL mapping, transcriptomics and metabolomics can be used to gain a more comprehensive understanding of mechanisms of resistance in plants and can therefore be used to further explore plant resistance to WSS (Biyiklioglu et al., 2018; Broekgaarden et al., 2018; Dhokane et al., 2016).

Research Objectives

This dissertation will comparatively assess molecular and genetic mechanisms of host plant resistance to WSS in cereal crops in a four-pronged approach:

1. Discover potential mechanisms that are involved in the resistance of oat to WSS through a comparison of small molecule profiles in infested oat and wheat plants
2. Compare small molecule profiles of pairs of NILs to identify pathways that may be involved in resistance associated with the 3B QTL in spring wheat and the 3A QTL in durum wheat
3. Analyze gene transcripts from the NILs in part 2 to obtain evidence for confirming which specific genes are responsible for the resistant phenotypes that have been observed in previous studies
4. Identify physiological differences and differences in volatile organic compounds (VOCs) between the NILs in part 3 at different growth stages to determine potential influences on host plant resistance and insect behavior.

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CHAPTER TWO

UNTARGETED METABOLOMICS PROFILING OF OAT (*Avena sativa* L.) AND WHEAT (*Triticum aestivum* L.) INFESTED WITH WHEAT STEM SAWFLY (*Cephus cinctus* Norton) REVEALS SPECIES DIFFERENCES ASSOCIATED WITH PLANT DEFENSE AND INSECT NUTRITION

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Manuscript Information Page

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Frontiers in Plant Science
Status of Manuscript:
_X_ Prepared for submission to a peer-reviewed journal
___Officially submitted to a peer-review journal
___Accepted by a peer-reviewed journal
___Published in a peer-reviewed journal

Abstract

Wheat stem sawfly (WSS), *Cephus cinctus* Norton, is a major pest of common bread wheat (*Triticum aestivum* L.) and other cultivated cereals in North America. Planting of cultivars with solid stems has been the primary management strategy to prevent yield loss due to WSS infestation, however expression of this phenotype can vary depending on environmental conditions and solid stems hinder biological control of WSS via braconid parasitoids *Bracon cephi* (Gahan) and *Bracon lissogaster* Muesebeck. This highlights the need for exploring additional sources of resistance outside of the historic solid stem phenotype. In the hollow stems of oat (*Avena sativa L.*), WSS larvae experience 100% mortality before they reach late instars, but the mechanisms for this observed resistance have not been characterized. Here, we use an untargeted metabolomics approach to examine the response of the metabolome of two cultivars of oat and four cultivars of wheat to infestation by WSS. Differentially expressed metabolites were identified between oat and wheat which were associated with the phenylpropanoid pathway, phospholipid biosynthesis and signaling, the salicylic acid signaling pathway, IAA degradation, and biosynthesis of 1,4-benzoxazin-3-ones (Bxs). Several phospho- and galacto-lipids were found in higher abundance in oat, and with the exception of early stem solidness cultivar Conan, both species experienced a decrease in abundance once infested. In all wheat cultivars except Conan, an increase in abundance was observed for Bxs HDMDB-glc and DIBOA-β-D-glucoside after infestation, indicating that this pathway is involved in wheat response to infestation in both solid and hollow stemmed cultivars. Differences between species in compounds involved in IAA biosynthesis, degradation and inactivation suggest that wheat may respond to infestation by inactivating IAA or altering the IAA pool in stem tissue. We

propose that the species differences found here likely affect the survival of WSS larvae and may also be associated with differences in stem architecture at the molecular level. Our findings suggest pathways to focus on for future studies in elucidating plant response to WSS infestation.

Introduction

The wheat stem sawfly (WSS), Hymenoptera *Cephus cinctus* Norton, is a major pest of grasses in the Great Plains of North America. Adult female WSS use their saw-like ovipositor to lay their eggs in the lumen of stems and once hatched, larvae begin consuming parenchyma tissue in the stem lining. As the larvae grows it breaches nodes, damaging vascular tissues, impacting photosynthetic ability and leading to decreased head weight (Delaney et al., 2010; Macedo et al., 2005). The stem girdling action of mature larvae to prepare overwintering chambers also weakens standing stems, which makes them prone to lodging and thus makes recovery of heads difficult during harvest (Ainslie, 1920; Morrill et al., 1998; Sherman et al., 2010). The stem-boring activity of the WSS larval stage causes devastating yield loss in cultivated grass hosts such as spring and winter wheat (*Triticum aestivum* L.). However, when eggs are laid in the stems of wild oat (*Avena fatua* L.) and cultivated oat (*Avena sativa* L.), larvae are unable to complete development (Criddle, 1917, 1923; Farstad, 1940; Roemhild, 1954; Sing, 2002). Although cultivated oat exhibits total resistance to WSS, the mechanism behind this resistance remains unknown (Weaver et al., 2004).

In spring wheat, the solid stem phenotype is utilized by producers as the primary defense against WSS, but it does not prevent infestation or stem cutting completely and may decrease success of biological control efforts (Rand et al., 2020; Rand et al., 2012). Solid stems are filled with pith which appears to hinder oviposition, larval growth and maturation. Solid stems do not

completely inhibit WSS and stem cutting as high as 30% has been reported for solid and semi-solid stemmed spring wheat cultivars (Sherman et al., 2010). While stem solidness in spring and winter wheat is a relatively stable phenotype across environments (Subedi et al., 2021), it can be less effective in preventing sawfly damage depending on light quality and sowing density (Beres et al., 2017; Nilsen et al., 2016; Nilsen et al., 2017; Roemhild, 1954). There is also evidence that braconid parasitoids *Bracon cephi* (Gahan) and *Bracon lissogaster* Muesebeck (Hymenoptera: Braconidae) cause less mortality to WSS larvae in solid stemmed cultivars compared to their hollow stemmed counterparts, indicating that continued use of solid stemmed cultivars may have a negative effect on populations of these parasitoids over time (Buteler et al., 2015b; Rand et al., 2020; Rand et al., 2012). Host plant resistance against a specific pest can also promote secondary pest populations over time (Straub et al., 2020). It is important to support the tritrophic system of plant, WSS and parasitoid to increase success of control efforts, and it is therefore necessary to continue to explore sources of resistance in addition to the solid stem phenotype.

The stem solidness trait is highly heritable and is associated with the quantitative trait locus (QTL) *Qss.msub-3BL* (Cook et al., 2004). This QTL has been well studied, and several alleles conferring differing levels of stem solidness have been identified at this locus (Cook et al., 2019; Varella et al., 2017; Wong et al., 2022). The allele *Qss.msub-3BLa* is associated with the hollow stemmed phenotype, while the allele *Qss.msub-3BLb* confers stem solidness throughout plant development. A third allele, *Qss.msub-3BLc* was identified in the cultivar Conan, which has solid stems early in development with pith disappearing as the plant matures. WSS is known to target many grass species in addition to wheat including barley, oat and many native or introduced grasses, all of which have a hollow stemmed phenotype (Achhami et al.,

2020; Cockrell et al., 2017; Criddle, 1917; Wallace & McNeal, 1966). Oat offers an opportunity to explore a source of complete resistance to WSS that appears unrelated to the stem solidness phenotype.

Female WSS will oviposit in wild oat as well as both small and large stemmed cultivated oat, but the larvae always die in the stem before reaching maturity (Criddle, 1917; Farstad, 1940; Holmes & Peterson, 1960; Holmes & Peterson, 1964; Sing, 2002; Wallace & McNeal, 1966; Weaver et al., 2004). This phenomenon has been discussed and documented for many years, and although research has explored the possibility of resistance due to physical differences between wheat and oat stems, the exact cause of larval mortality in oat remains unknown. In 1923, Criddle described “excessive sap” in oat stems as a possible cause of larval mortality, but this is likely due to environmental conditions as it was not replicable in dry environments (Criddle, 1923; Farstad, 1940). Though the outcome is likely not caused by free moisture, there is some evidence that larvae in oat stems do not begin development as readily as larvae in wheat. In oat, larvae are less likely to form extensive, continuous tunnels throughout the stem and they molt less frequently than larvae developing in wheat (Farstad, 1940). Past research on oat plant responses to WSS infestation and injury has focused on the possibility of resistance due to physical differences between wheat and oat stems (Roemhild, 1954). Tissue surrounding vascular bundles in the nodes of oat stems is composed of tough sclerenchyma tissue, as opposed to the more pliable parenchyma tissue found in wheat nodes. However, this structural difference is not enough to explain the resistance observed in oat, since some larvae are still able to penetrate multiple nodes as they move throughout the stem (Roemhild, 1954). These observations on larvae feeding on oat stem tissues seem to suggest that there may be nutritional

deficiencies or perhaps more likely, compounds present in oat with antifeedant or insecticidal activities. While little is known about the identity of compounds in oat which are detrimental to WSS development, it has been determined that stems of oat and wheat do not differ in moisture or nitrogen content at earlier growth stages when WSS larvae are susceptible to mortality (McGinnis & Kasting, 1961). Additionally, both oat and wild oat are known to produce several active phenolic compounds in roots, shoots and seeds (Kato-Noguchi et al., 1994; Schumacher et al., 1983). In early development these are exuded allelopathic compounds, preventing establishment of seedlings of other species, and can also be induced in response to pest or pathogen attack at any growth stage (Kato-Noguchi et al., 1994; Pérez & Ormeño-Nuñez, 1991) . Overall, little mechanistic research has been done to explore oat resistance to WSS and defining the potential molecular mechanisms of WSS resistance in oat represents an important step towards developing wheat cultivars with new forms of resistance to WSS.

In this study we use liquid-chromatography mass spectrometry (LC-MS) to evaluate the metabolites that define the physiological response of spring wheat and oat to WSS infestation. Montana spring wheat cultivars Choteau, Scholar, Conan and Reeder were chosen for comparison against the oat cultivars Dane and Otana. The cultivars Choteau and Scholar are considered very or somewhat resistant to WSS, respectively, and both have the allele *Qss.msub-3BLb* associated with the solid stem phenotype, while Reeder is a susceptible hollow stemmed cultivar with the allele *Qss.msub-3BLa*. Conan shows a unique phenotypic expression of stem solidness, being solid at early growth stages and losing pith as the plant matures (Sherman et al., 2010; Talbert et al., 2014; Varella, 2016). The goal of this research is to discover mechanisms

that are involved in the resistance of oat to WSS through a comparison of the small molecule profiles of infested oat and wheat plants with varying degrees of resistance.

Materials and Methods

Experimental Conditions and Plant Collection.

Seeds were planted in a mix of MSU mix and Sunshine mix #1 in a 50:50 by volume ratio. MSU mix contained a composite of mineral soils from the Gallatin Valley, Canadian sphagnum peat moss and washed concrete sand in a 1:1:1 by volume ratio as in Varella et al. (2017). Sunshine Mix #1 consisted of a soil-less blend of Canadian Sphagnum peat moss and horticultural grade Perlite. Seeds were planted in a greenhouse at the Plant Growth Center at Montana State University. For each cultivar three pots containing four seeds were planted for infestation and two pots also containing four seeds were planted for controls. Plants were infested when the first internode was detected, at approximately Zadoks growth stage 32 (Zadoks et al., 1974). A cage with 530 µM mesh openings was placed over the entire plant and secured using a stake and wire. A 50/50 soil mix was added to the base of the chamber to prevent WSS escape. Supplementary lighting was placed approximately 45 cm on either side of the chamber to ensure that the sawflies were active. Three WSS adult females were introduced to each chamber and oviposition was allowed for 72 hours, after which the chambers and any remaining sawflies were removed. Plant tissue was collected two weeks after the introduction of WSS. To process samples, the plant was cut at ground level and only the top two internodes were separated from the plant using a razor blade to ensure that stem tissue collected from infested plants was free of frass. The leaves were separated from the internodes, and internodes were wrapped in aluminum foil and immediately flash frozen in liquid nitrogen. Samples were considered infested if frass or

larvae were observed in the lower internodes. All sample processing was done in 60 seconds or less. Samples were then stored in -80°C until metabolites were extracted.

Sample Processing for Metabolomics.

Frozen wheat and oat stems were ground in liquid nitrogen with a mortar and pestle. Stem powder (approximately 150 mg per sample) was immersed in 100% methanol (MeOH) at 70°C for 15 min. Samples were vortexed for 1 min and then centrifuged (25,000 g, 10 min, 4°C). Proteins were separated from the metabolites by an acetone precipitation (two and a half parts acetone to one part tissue solution) at -80°C overnight, followed by centrifugation (25,000 g) at 4°C for 10 min. The resulting supernatant fraction was dried in a speed vacuum (low heat setting) and stored at -80°C. Prior to analyses by liquid chromatography-mass spectrometry (LC-MS), samples were resuspended in 20 µL of 50% HPLC grade water / 50% MeOH.

LC-MS.

Metabolite analysis was conducted at the Montana State Mass Spectrometry Facility using an Agilent 1290 ultra-performance liquid chromatography (UPLC) interface (Agilent Technologies, Santa Clara, CA, USA) fitted to an Agilent 6538 Accurate-Mass quadrupole time-of-flight mass spectrometer. Metabolites were separated by reversed-phase (RP) chromatography on a Kinetex 1.7 µm C18 150 x 2.1 mm column (Phenomenex, Torrance, CA) kept at 50°C with a flow rate of 600 µL min -1. The elution profile implemented started with a two-minute step of 98% solvent A (0.1% formic acid in H2O; waste) with 2% solvent B (0.1% formic acid in acetonitrile) followed by a 2% to 95% solvent B gradient over 24 min, a continued 95% solvent B for two min, and then a return to 2% solvent B over two min.

Mass detection was performed in positive mode, with a cone voltage of 3,500 V and a fragmentor voltage of 120 V. Drying gas temperature was 350°C (flow of 12 L per min -1) and the nebulizer was set at ~5.2 bar. Data was acquired with the following parameters: mass-to-charge ratio (m/z) range of 50-1,000 at 25,200 m/z-s. Mass analyzer resolution was 18,000 and post calibration tests had mass accuracy of approximately one ppm. For MS/MS acquisition, both standard compounds and ions of interest within a tolerance window of 1.7 m/z units were fragmented at 10 and 20 V.

LC-MS Data Preprocessing and Analysis.

Raw LC-MS data was converted to mzXML files using MSconvert from Proteowizard (Chambers et al., 2012). MZmine2 was used for preprocessing of spectral data (Pluskal et al., 2010). Raw data was acquired in centroid mode and was assigned m/z and retention time values using mass detection with noise level set at 1.0 E 3. Chromatograms were built using the chromatogram builder setting with minimum time span for peaks set to 0.1 min, minimum peak height set to 1.0 E 3 and m/z tolerance between 0.01 and 30 ppm. Resulting peaks were normalized using the same m/z tolerance, a retention time of 0.25 min and a minimum standard intensity of 2.0 E 4. Normalized peaks were aligned, and the gap-filling feature was used to fill in missing peaks. Metabolites were tentatively identified using the online database metacyc.org, followed by classification using the Chemical Entities of Biological Interest database (ChEBI) (Caspi et al., 2014; Katajamaa & Orešič, 2007; Pluskal et al., 2010). (Caspi et. al., 2014; Hastings et al. 2015; Katajamaa & Orešič, 2007; Pluskal et al., 2010).

Statistical Analysis.

Data pre-processing and analysis was performed in R version 3.5.1 (R Core Team, 2022) using the R package MetaboAnalystR and ggplot2 (Pang et al., 2020, Wickham 2016). Identified features were log transformed and range scaled to meet the assumption of normality. Normalized values were used to perform a principal component analysis (PCA) to identify which features contributed most to the variability of the dataset. A two-way ANOVA was also performed with infestation status and oat/wheat cultivar as fixed effects to identify whether there were differences in the metabolic profiles of infested and control oat and wheat plants. To further explore the response to infestation by the individual species, the oat and wheat data was separated into two datasets. These datasets were used to perform PCA as well as two-way ANOVA with infestation status and cultivar as fixed effects. For all ANOVA testing, post-hoc pairwise testing was performed using Fishers Least Significant Difference (LSD) test and false discovery rate (FDR) correction of p-values was used to correct for multiple comparisons.

Results

Complete Dataset – Oat and Wheat.

PCA utilizing all features from the oat and wheat dataset showed clear separation of the oat and wheat samples based on the first principal component (PC1) which explained 45.7% of the variability in the dataset. The second principal component (PC2) explained 9.1% of the variability and was able to clearly discriminate between the oat cultivars. Some separation of the infested and control wheat samples was also observed based on PC2, with the exception of Conan samples which showed high variability in infested and control samples (Figure 2.1).

Two-way ANOVA of all oat and wheat samples identified 160 features that showed significant differences between oat and wheat cultivars (p-value<0.05) (Table 2.1). Differences in eight compounds, benzoyl-β-D-glucopyranose, hemigossypol, salicylate 2-O-β-D-glucoside, serotonin, xanthine, 2-omega-hydroxy-C22:0-LPA, dihydroxyferuloyl-sinapoyl spermidine and indole-3-acetyl-isoleucine, were observed based on infestation status (p-value<0.05). There was evidence for an interaction between infestation status and cultivar for benzoyl-β-D-glucopyranose, 10-deacetyl-2-debenzoylbaccatin III, vitexin-2-O-β-D glucoside, tetrahydropteroyl-tri-L-glutamate, phlorizin and 7-hydroxyflavone. Of these features, it was possible to classify 141 and match 126 to their respective biological pathways. The majority of features were found to be directly involved in plant defense, including several related to glucosinolate biosynthesis and degradation (Figure 2.2). Groups of terpenoids, phenols, alkaloids, glycerolipids and phospholipids were also significantly higher in oat samples compared to wheat in both infested and control groups (p-values<0.05) (Figure 2.3).

In the complete dataset, O-sinapoylglucarate, O-sinapoylglucarolactone and 2-O-caffeoylglucarate were all differentially expressed between oat and wheat samples (p-value<0.001, Figure 2.4). Additionally, abundance of 16-feruloyloxypalmitate, oleate, and 5-hydroxyconiferyl alcohol were significantly lower in wheat compared to all oat cultivars (p-value<0.001, Figure 2.5). Several phospho- and galacto- lipids involved in lipid biosynthesis, degradation and signaling pathways were found to be significantly higher in oat, but there was no effect of infestation status on the abundance of lipids in this group (Figure 2.6, Figure 2.7). Cultivar had a strong effect on the abundance of salicylic acid-related compounds salicylate 2-O-β-D-glucoside, salicin and salicyl-6-hydroxy-2-cyclohexene-on-oyl (p-values<0.05), while in

infested Dane samples, infestation status had an effect on the levels of salicylate 2-O-β-D-glucoside (Figure 2.8). Cultivar had a strong effect on the abundance of several IAA-derived compounds, while infestation status had no significant effect on the abundance of these compounds. Abundance of 3-hydroxy-2-oxindole-3-acetyl-aspartate was significantly higher in oat samples, while 2-oxindole-3-acetyl-L-aspartate, indole 3 acetyl glutamate, and indole-3-acetyl-leucine had significantly lower abundance in oat samples compared to wheat (p-values<0.001, Figure 2.9).

Oat Dataset.

PCA using the oat dataset did not show any separation based on infestation status. The first principal component (PC1) explained 34.8% of the variability in the dataset and also allowed for discrimination of cultivars, while the second principal component (PC2) explained 15.7% of the variability in the dataset (Figure 2.10).

Two-way ANOVA of oat samples identified 97 features that were significantly different between cultivars (p-value<0.05) (Table 2.2). Of these features it was possible to classify 81 and match 79 to their respective biological pathway. Features identified as glucosyl-limonin, 6-hydroxy-allocryptopine and indole-3-acetohydroximoyl-glutathione were significantly higher in ‘Dane’ control samples compared to ‘Otana’ control samples (p-value<0.05), with this difference decreasing significantly upon infestation (p-value <0.05). Benzoyl-β-D-glucopyranose showed an opposite expression pattern, with a significant difference only observed between the cultivars in infested samples (p-value<0.05). For glucosyl-limonin and 6-hydroxy-allocryptopine there was strong evidence of an interaction between cultivar and infestation status (p-value<0.05), while for indole-3-acetohydroximoyl-glutathione and benzoyl-β-D-glucopyranose, evidence of

an interaction was weak (p-value=0.05). All other features were not significantly different between infested and control samples but did differ significantly between cultivars (p-value<0.05). The majority of these features were involved in plant defense or biosynthetic pathways including nucleotide cycling and degradation, phosphatidylcholine biosynthesis and biosynthesis of secondary metabolites (Figure 2.11). Features that were significantly different between the oat cultivars included several alkaloids, phenols, terpenes, flavonoids and carbohydrates (Figure 2.12).

Wheat Dataset.

PCA with only the wheat dataset showed Choteau, Conan, Reeder and Scholar samples forming distinct clusters, but overlap between groups was observed due to the variability within sample groups (Figure 2.13). The first principal component (PC1) explained 19.7% of the variability in the dataset while the second principal component (PC2) explained 16.3%. Overall, Conan samples showed the tightest clustering, with lower variability observed in infested and control groups when compared with the other cultivars.

In the two-way ANOVA of wheat samples, a total of 58 features showed significant differences. Cultivar had a significant effect on the abundance of 52 features and infestation status had a significant effect on 11 features, with evidence for an interaction between cultivar and infestation status for three features (p-value<0.05) (Table 2.3). Of the 58 total features, it was possible to classify 52 and assign 39 to their respective pathway (Figure 2.14, Figure 2.15). Luteolin 7-O-β-D-glucuronide, hemigossypol, 2-omega-hydroxy-C22:0-lysophosphatidic acid, serotonin, HMDBOA glucoside, dihydrocurcumin, 7-deoxyloganetate, xanthine, bis(β-D-glucosyl) crocetin, laudanosine and bornyl diphosphate were significantly different between

infested and control samples in wheat (p-value<0.05). However, there was also evidence of an interaction between cultivar and infestation status for laudanosine, luteolin 7-O-β-D-glucuronide, and vitexin 2-O-β-D glucoside (p-value<0.05). For 2-omega-hydroxy-C22:0-lysophosphatidic acid, variability within cultivars was much higher in control samples compared to infested samples, while for serotonin and bis(β-D-glucosyl) crocetin, variability increased upon infestation. Conan showed no change in dihydrocurcumin, hemigossypol, 7-deoxyloganetate, HMDBOA glucoside, xanthine or laudanosine between infested and control samples. In Choteau, abundance of hemigossypol, 7-deoxyloganetate, serotonin, HMDBOA glucoside, xanthine, bis(β-D-glucosyl) crocetin, bornyl diphosphate and laudanosine were higher in infested samples compared to uninfested samples. Abundance of luteolin 7-O-β-D-glucuronide, dihydrocurcumin and 2-omega-hydroxy-C22:0-lysophosphatidic acid decreased in infested Reeder samples, while hemigossypol, bis(β-D-glucosyl) crocetin, xanthine, HMDBOA glucoside, and bornyl diphosphate decreased upon infestation in Reeder. In Scholar, abundance of luteolin 7-O-β-D-glucuronide, 2-omega-hydroxy-C22:0-lysophosphatidic acid, hemigossypol, bis(β-D-glucosyl) crocetin, xanthine, HMDBOA glucoside, bornyl diphosphate, 7-deoxyloganetate and serotonin were all found in higher abundance in infested samples. Infestation status and cultivar both had a significant effect on the abundance of HDMBOA-Glc in the wheat dataset, although there was no evidence for an interaction between infestation status and cultivar (p-values<0.05, Figure 2.16).

Discussion

Phenylpropanoid Pathway and Species Differences in Lignin and Suberin Biosynthetic Pathways.

The phenylpropanoid pathway is extensive and complex, producing a wide range of compounds which are involved in plant defense in several ways including systemic signaling and providing chemical or physical barriers to insect or pathogen attack. This pathway is responsible for the biosynthesis of hydroxycinnamic acids, flavonoids, isoflavanones, anthocyanins, and sinapate esters (Strack et al., 1987b). Several structural biopolymers are derived from the phenylpropanoid pathway as well, including lignin and suberin. Production of these compounds is highly variable depending on species and nearly every class of molecule produced by this superpathway has functions that are either directly or indirectly involved in plant defense (Dixon et al., 2002a).

Biosynthesis of hydroxycinnamate (HCA) esters likely proceeds via three unique routes: one utilizing HCA-CoA thioesters, another using HCA-glucosides from sinapate ester biosynthesis and the third utilizing HCA-quinate (Strack et al., 1988; Strack et al., 1987b). O-sinapoylglucarate and O-sinapoylglucarolactone, both products of the second route of HCA ester biosynthesis, were found in lower abundance in oat samples when compared to wheat samples. In wheat, abundance of O-sinapoylglucarate and O-sinapoylglucarolactone increased or stayed the same when plants were infested, while infested oat plants had a significant decrease in O-sinapoylglucarolactone compared to uninfested oat plants (Figure 4). Overall, 2-O-caffeoylglucarate, a product of the third route of HCA ester biosynthesis, had increased abundance in oat samples compared to wheat and also had significantly lower abundance in infested oat plants compared to uninfested oat plants (Figure 4). These results indicate that oat

may produce HCA esters primarily via the HCA-quinate route. Greater abundance of L-quinate in oat was observed, but the difference compared to wheat was not found to be statistically significant (Data not shown). This supports the suggestion that the HCA-quinate route of HCA biosynthesis is upregulated in oat, since the last step in biosynthesis of this compound produces L-quinate. It is not known whether the routes to HCA production differ in terms of efficiency or speed of response, so it is difficult to determine what role the HCA-quinate route plays in oat resistance to larval feeding.

Hydroxycinnamic acid esters in plant tissues have a direct effect on plant resistance and can decrease oviposition and have a negative effect on larval mortality (Anyanga et al., 2021). They can also form polymers with polyamines to form hydroxycinnamic acid amines (HCAAs) which are deposited in the cell wall near regions of pathogen infection or wounding by boring insects, a process that is associated with strengthening of cell walls and increased resistance (Barros-Rios et al., 2011; Facchini et al., 2002; Grandmaison et al., 1993; Gunnaiah et al., 2012). HCAAs themselves can influence cell growth and lignin content as well as lignin subunit composition, which also affects plant resistance (Lima et al., 2013).

Suberin and lignin are structural polymers that are essential for plant protection from infection by fungal pathogens or infestation by insects (Bakaze et al., 2020; Kosma et al., 2010; Li et al., 2007; Wainhouse et al., 1990). Infection by some pathogens induce reinforcement of vascular cell walls with a ligno-suberin coating that strengthens the cell wall, preventing degradation and restricting the movement of the pathogen, conferring resistance (Kashyap et al., 2022). In wheat, lignin is present in the parenchyma of solid-stemmed cultivars, while lignification is not as extensive in barley or oat (Roemhild, 1954). In our complete dataset,

abundance of 16-feruloyloxypalmitate, oleate, and 5-hydroxyconiferyl alcohol was significantly lower in wheat compared to all oat cultivars (Figure 5). 16-feruloyloxypalmitate and oleate are predicted to be involved in suberin biosynthesis, while 5-hydroxyconiferyl alcohol is incorporated into lignin via a dehydrogenation reaction, forming lignins that are linear in nature (Elder et al., 2016). While several studies have confirmed the positive impact of increased lignin on plant resistance to fungal pathogens and infestation by insects (Bi et al., 2010; Wainhouse et al., 1990), lignin content has not been found to have any effect on the amount of sawfly tunneling or stem cutting in the sawfly system (McNeal et al., 1965).

However, total lignin content is only one aspect of mechanical resistance to pests and pathogens. Lignin is a polymer composed of syringyl (S), guaiacyl (G) and p-hydroxyphenyl (H) subunits which may have an effect on resistance. In particular, some research has explored the influence of lignin subunit composition (S/G ratio) on palatability of plant tissue to insect herbivores, and there is evidence that resistant plants exhibit an altered S/G ratio depending on species (Kärkönen et al., 2014; Poerschmann et al., 2005; Sewalt et al., 1997). Lignin formed in response to bacterial infection or insect attack contains less S monolignin and more G and H monolignins, illustrating that biosynthesis of lignin can change depending on type of stress, though this does not always coincide with increased plant resistance (Buhl et al., 2017; Zhang et al., 2007). Further study is necessary to elucidate whether the lignin composition of oat differs from that of wheat and whether this has an effect on WSS larval feeding or survival in oat.

Phospholipids in oat and wheat.

Phospholipids such as phosphatidylcholine and phosphatidylethanalomine are important structural lipids found in plant cell membranes. These molecules can also be hydrolyzed by

phospholipases and serve as co-factors, signaling molecules or precursors to signaling molecules that are necessary for plant defense responses (Laxalt & Munnik, 2002; Munnik et al., 1998). Phospholipids, galactolipids and related molecules found in this study have the potential for involvement in plant defense responses in some capacity and may be linked to the same putative pathway (Figure 6). Several phospholipid metabolizing enzymes have been identified and are thought to play an important role in plant defense signal transduction. A known product of phospholipases C (PLC; EC 3.1.4.3) and D (PLD; EC 3.1.4.4) is phosphatidic acid which is a conjugate acid of phosphatidate (Liscovitch et al., 2000). In many plants, levels of phosphatidic acid have been shown to increase in response to wounding, drought stress or treatment with a general synthetic elicitor (Frank et al., 2000; Lee et al., 1997; van der Luit et al., 2000). Phosphatidic acid is also a precursor to many complex lipids including digalactosyldiacylglycerol, which in turn contributes to salicylic acid biosynthesis and systemic acquired resistance (SAR) signal azelaic acid (Gao et al., 2014). Phosphatidic acid can also activate the NADPH oxidase complex, indirectly triggering an oxidative burst in response to pathogen attack (Laxalt & Munnik, 2002).

In this study, both species experienced a decrease in lipid abundance in response to infestation, with the exception of Conan, which showed an increase in concentration for the majority of lipids observed. Lipid components of plant cellular membranes, putatively identified as 1-18:1-2-18:1-phosphatidylethanolamine, 1-18:0-2-18:1-phosphatidylethanolamine and 1-18:3-2-trans-16:1-phosphatidylglycerol, were significantly greater in oat compared with all cultivars of wheat. In the oat dataset, possible free galactolipids 1-18:2-2-18:2-digalactosyldiacylglycerol and 1-18:3-2-18:2-monogalactosyldiacylglycerol both decreased in

response to infestation, but only cultivar had a significant effect on abundance. Lipids, including phospholipids, are nutritionally significant to many insects and are often required for normal growth and development (Turunen, 1979). Conversely, high concentrations of lipids in plant tissues can also cause larval mortality and decreased larval growth in some species (Li et al., 2014; Liao et al., 2021). WSS larvae are adapted to feeding inside the plant stem, making it difficult to test their dietary requirements with artificial diets, so little is known about their specific dietary requirements, but it is possible that higher levels of lipids in oat may have a negative effect on larval growth and development (Macedo et al 2005).

Phospholipids have been well studied in relation to wheat resistance to Hessian fly, an insect that feeds within the exterior of the stem wall. In several studies using wheat lines resistant to Hessian fly, a decrease in membrane lipid concentration and increase in fatty acids and their derivatives has often been observed in response to feeding by Hessian fly larvae (Khajuria et al., 2013; Zhu et al., 2012a). In our results, infestation did not have a significant effect on the levels of phospho- or galactolipids in any of the datasets, although a decrease of abundance was observed in infested samples, indicating that phospholipid signaling is likely not a strong response to WSS infestation in either oat or wheat (Figure 7). The resistant cultivar Conan had similar levels of structural lipids to the other wheat cultivars but had increased abundance of free lipids 1-18:2-2-18:2-monogalactosyldiacylglycerol, 1-18:2-2-18:2-digalactosyldiacylglycerol, 1-18:2-2-18:3-digalactosyldiacylglycerol and 1-18:1-2-18:1-phosphatidate, though this increase was not significant in either the complete or wheat-only dataset (Figure 7). These results suggest that the increase in free lipids might be associated with the disappearance of pith observed as Conan matures and indicate that Conan may be more efficient than other wheat cultivars at re-

mobilizing structural lipids after degradation to signaling lipids or precursors. The downstream effects of increased phosphatidic acid, including SAR and induction of an oxidative burst, may also partially explain the resistance of Conan to sawfly damage.

Salicylic Acid and Related Metabolites.

Salicylic acid (SA) and jasmonic acid (JA) hormone signaling pathways are the two most important and well-studied in relation to induced plant defense (Erb et al., 2012; Howe & Jander, 2008). The crosstalk between these pathways is complex, but SA can have a negative effect on JA-induced resistance, making plants more susceptible to damage from insect herbivores (Bodenhausen & Reymond, 2007; Bruessow et al., 2010; Stotz et al., 2002). Salicylates such as salicin and salicortin also play a role in direct plant defense against herbivory, negatively affecting larval performance by decreasing growth rates (Hemming & Lindroth, 2000; Lindroth & Bloomer, 1991; Lindroth & Peterson, 1988; Zhang et al., 2013). Additionally, there is evidence that activation of jasmonate and salicylic acid defenses can increase parasitism or predation of herbivores in many plant species (Dicke et al., 1990; Thaler, 1999; van Poecke & Dicke, 2002).

In this study, there was a strong effect of cultivar on the abundance of the salicylic acid-related compounds salicylate 2-O-β-D-glucoside, salicin and salicyl-6-hydroxy-2-cyclohexene-on-oyl, while infestation status only had an effect on the levels of salicylate 2-O-β-D-glucoside in infested Dane samples. There was no significant effect of cultivar or infestation status on the abundance of salicortin, however, salicyl-6-hydroxy-2-cyclohexene-on-oyl, the final precursor in salicortin biosynthesis was significantly higher in both oat cultivars (Figure 8). Salicortin itself did decrease in all cultivars of oat and wheat in response to infestation, but this response was not

found to be significant. Regardless, as salicyl-6-hydroxy-2-cyclohexene-on-oyl is likely more stable than salicortin *in vivo*, the increased abundance of salicyl-6-hydroxy-2-cyclohexene-on-oyl may indicate an upregulation of the salicortin biosynthesis pathway in oat. In a study of willow *Salix sericea* (Marsh.) subjected to damage by herbivory from various species of beetle, salicortin decreased in the affected leaves four days after the start of damage, possibly due to consumption of leaf tissue high in this compound (Fields & Orians, 2006). Since the tissue collected for our study was undamaged by larval feeding and was collected after larvae were able to feed within the stem for some time, the decrease in salicortin is more likely due to a systemic response or degradation as opposed to a short term induced defense response. Salicortin may be remobilized to damaged regions of the stem where it is rapidly degraded into salicin during larval feeding, which might explain the decrease in levels of salicortin in systemic stem tissue of infested samples.

In the complete dataset, cultivar had an effect on abundance of only one compound derived from the jasmonates biosynthesis pathway, 12-hydroxyjasmonic acid 12-O-β-D-glucoside (tuberonic acid glucoside, TAG), while infestation status had no measureable effect. Two additional jasmonate-related compounds, jasmonoyl-CoA and jasmonoyl-L-isoleucine, were identified in the complete dataset, but cultivar and infestation status had no effect on abundance (data not shown). While not a significant result, TAG decreased with infestation in Choteau and Conan with no change in infested Scholar or Reeder. Conversely, both cultivars of oat showed an increase in TAG abundance in infested samples, although this result was not significant in the complete or oat dataset. After JA is converted to TAG in damaged regions of the plant, it is translocated to undamaged plant tissue where it contributes to down-regulation of

genes involved in JA biosynthesis, which may explain the insignificant effect of WSS infestation on JA-associated compounds identified in our analysis (Gidda et al., 2003; Seto et al., 2009).

DIMBOA Pathway.

2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and related 1,4-benzoxazin-3-ones (Bxs) are related to the resistance of cereal species to insects, fungi and bacteria (Niemeyer, 1988). These metabolites are present in plant cells as glucosides, which become available to hydrolyzing enzymes once plant tissues are damaged by insect feeding (Virtanen & Hietala, 1960). Bxs have also recently been discovered to play a key role in the stem elongation phase in winter wheat (Xu et al., 2021). Much of the research conducted on this class of molecules has focused on increased levels of DIMBOA-Glc, 2-(2-hydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (HDMBOA-Glc) and 6-methoxy-2-benzoxazolinone (MBOA) in maize resistance to European corn borer, *Ostrinia nubilalis*, however Bxs have also been found to be involved in plant resistance to several species of aphids, as well as larvae from many species (Campos et al., 1989; Houseman et al., 1992; Niemeyer et al., 1989; Ortego et al., 1998). Evidence from previous untargeted proteomics and metabolomics experiments show that Bxs may also be involved in plant response to WSS in spring and winter wheat (Biyiklioglu et al., 2018; Lavergne et al., 2020).

A metabolite putatively identified as DIMBOA was included in our analysis, but abundance was only affected by cultivar in the oat dataset with no differences in the wheat or complete datasets. While all wheat cultivars experienced an increase in abundance of HDMBOA-Glc after infestation, the increase in Conan was insignificant compared to Choteau, Scholar and Reeder (Figure 16). HDMBOA-Glc can be induced during feeding by *O. nubilalis* or

upon treatment with jasmonic acid (JA) or chitin derived saccharides in wheat and maize (Dafoe et al., 2011; Oikawa et al., 2001; Oikawa et al., 2002). After hydrolysis of HDMBOA-Glc, HDMBOA decomposes rapidly into MBOA at a faster rate than other known Bxs, and both HDMBOA and MBOA have negative effects on larval growth of Southwestern corn borer, *Diatraea grandiosella* Dyar (Grambow & Lückge, 1986; Hedin et al., 1993). This means that HDMBOA-Glc may be involved in the rapid defense response of wheat to WSS, although it appears that Conan may return to pre-infestation levels more quickly than other cultivars.

In our complete dataset, DIBOA-β-D-glucoside had significantly higher abundance in both oat cultivars compared to all wheat cultivars. While not significant, wheat cultivars experienced an increase in DIBOA-β-D-glucoside upon infestation while both oat cultivars showed a decrease of this metabolite after infestation (Figure 16). The increased abundance of DIBOA-β-D-glucoside in infested wheat samples observed here matches the plant response observed in the hollow-stemmed partially tolerant cultivar Hatcher reported in figures of a previous study of plant metabolomic response to WSS (Lavergne et al., 2020). Conversely, we observed a decrease in DIBOA-β-D-glucoside upon infestation in all oat cultivars. In a separate study of the proteome of infested spring wheat cultivars, downregulation of DIMBOA glucoside β-D-glucosidase, a protein which hydrolyzes DIMBOA-glucoside to DIMBOA and D-glucose, was identified in infested stems of Scholar, but no change was observed in resistant cultivar Choteau (Biyiklioglu et al., 2018). These conflicting results illustrate the complex involvement of this pathway in WSS resistance and are not unexpected, since Bxs vary significantly based on cultivar, growth stage, plant part sampled and time after infestation (Huang et al., 2006; Niemeyer et al., 1989). Adding to the complexity of this response, there is some evidence that

Bxs in the diet of cereal aphid species can have a positive effect on aphid predators, maximizing the tritrophic interaction between wheat, aphids and aphid predator *Eriopis connexa* Germar. (Martos et al., 1992). This means that Bxs in the diet of WSS larvae have the potential to impact the fitness of specialist braconid parasitoids that have WSS as their only known host. This possibility, along with the association of Bxs in plant response to WSS infestation and with the specific response of the resistant cultivar Conan show that further exploration of this pathway will be beneficial to understanding WSS resistance.

Indole-3-acetate (IAA) Degradation.

Phytohormone indole-3-acetic acid (IAA), is the primary auxin found in plants, and is involved in many aspects of plant development and response including tropism, cell elongation and stem growth, cell division, phloem and xylem differentiation, root initiation, flowering, senescence and abscission (Davies, 2010). Concentrations of IAA in plant tissues is regulated by the plant in several ways, including biosynthesis, degradation, activation, transport in and out of cells, and inactivation via formation of IAA conjugates (Bartel et al., 2001; Caruso, 1987). Despite knowledge of the existence of these regulatory mechanisms, the exact pathways for many are still only partially elucidated (Ljung, 2013). IAA can also be produced by some species of insects in high concentrations, allowing them to form galls, increase nutritive value of plant tissue or overcome structural plant defenses (Dafoe et al., 2013; Tooker & De Moraes, 2010, 2011).

In the complete dataset, cultivar had a strong effect on the abundance of several IAA-conjugates and IAA-derived compounds that are involved in indole-3-acetate degradation, while infestation status had no significant effect on the abundance of these compounds. Abundance of

2-oxindole-3-acetyl-L-aspartate, indole 3 acetyl glutamate, and indole-3-acetyl-leucine had significantly lower abundance in oat samples compared to wheat, while 3-hydroxy-2-oxindole-3-acetyl-aspartate was significantly higher in oat samples (Figure 9). The function of IAA-leucine appears to be temporary storage to supply free IAA when needed by the cell (for IAA-activation), so the lower abundance observed in oat may indicate that free IAA can be readily provided by degradation of IAA-leucine or IAA-glutamate in wheat (Woodward & Bartel, 2005). IAA-glutamate is also involved in an indole-3-acetate conjugate degradation pathway and may serve as a temporary storage form of IAA since it is easily reconverted to IAA in vivo (Hayashi et al., 2021). IAA-glutamate can also be irreversibly oxidized to 2-oxindole-3-acetyl-L-aspartate and further hydrolyzed to 2-oxindole-3-acetic acid, an inactive form of IAA (Hayashi et al., 2021). 33-hydroxy-2-oxindole-3-acetyl-aspartate was found in lower abundance in oat, with the exception of Dane infested samples. This compound may be involved in degradation of 2-oxindole-3-acetic acid aspartate to 3-O-β-glucosyl -2-oxindole-3-acetyl-aspartate, but it is unknown whether this mechanism can be reversed to provide free IAA (Caspi et al., 2014).

The significantly lower abundance of IAA-glutamate and the decrease that was observed in infested oat, while not significant, suggests that oat may not inactivate IAA to the same extent as in wheat, instead storing it in forms that can be re-activated. In fact, with the exception of Conan, all wheat cultivars experienced an increase in IAA-glutamate upon infestation and also had higher abundance of 2-oxindole-3-acetyl-L-aspartate, the product of the irreversible step of IAA inactivation in both infested and uninfested tissue samples. These results indicate that wheat may respond to WSS infestation by inactivating IAA in the stem, while fully irreversible inactivation of IAA is more variable depending on cultivar.

Free IAA, whether produced by the plant or by insect, can affect insect survival not only through physical consumption, but by altering plant host material for protection or increased nutrient availability. Hessian fly infestation causes elevated levels of IAA without a corresponding induction of jasmonic acid and salicylic acid, leading to increased nutrient availability while simultaneously lowering plant defense activation (Tooker & De Moraes, 2010). In maize, high levels of IAA are excreted in the frass of European corn borer, leading to increased protein accumulation in nearby tissues which provide larvae with more nutritional content, offsetting the decline in growth rates caused by the presence of increased plant defense compounds (Dafoe et al., 2013). IAA application to the larvae of lesser wax moth, *Achoria grisella* not only has an effect on lipid, protein and glycogen levels in the hemolymph of the pest, but also affects hemolymph content of its braconid natural enemy *Apanteles galleriae* (Uçkan et al., 2014). These changes can even have a negative effect on natural enemy populations, increasing adult emergence time after pupation and decreasing overall longevity of *A. galleriae* adults (Uçkan et al., 2011). These studies indicate that further exploration of IAA biosynthesis and degradation pathways may provide more information about the tritrophic interaction between wheat, WSS and their parasitoids.

Conclusion

The solid stem phenotype has historically been the most utilized defense against damage and yield loss from WSS infestation, but due to variability in solid stem expression under different growing conditions, and the negative effect of solid stems on braconid parasitoid populations, substantial stem cutting and yield loss can still occur. To support parasitoid populations and maximize the effectiveness of the tritrophic interaction between host, pest and

parasitoid, it is necessary to explore new sources of resistance which are not associated with solid stems. Oat exhibits complete resistance to WSS, causing 100% larval mortality to early instar larvae, which makes it a prime candidate for investigating new mechanisms of host plant resistance.

Here, we identified differentially expressed metabolites between cultivars of oat and wheat which were associated with changes to the phenylpropanoid pathway and phospholipid biosynthesis and signaling. Compounds such as O-sinapoylglucarate, O-sinapoylglucarolactone, 2-O-caffeoylglucarate (all are HCAs) and 5-hydroxyconiferyl alcohol are ubiquitous in plants and are generally related to cellular organization and tissue structure. Due to their presence in stem tissue, HCAs as well as membrane and free lipids are directly encountered by foraging females during ovipositor insertions as well as by larvae feeding inside the stem. HCAs can also affect oviposition behavior and larval survival by strengthening the cell wall and inducing changes in overall lignin content and composition, while lipids can serve as signaling molecules, affecting a wide range of downstream defense responses. Additionally, increased abundance of 5-hydroxyconiferyl alcohol in oat suggests that there may be differences in lignin subunit composition that is related to WSS resistance. In oat, several phospho- and galacto- lipids were also found in greater abundance, and both species experienced a decrease in phospholipid abundance after infestation, with the exception of Conan, which has the early stem-solidness phenotype and loses pith as the plant develops. Other studies have also identified a role for the phenylpropanoid pathway in plant defense against wheat stem sawfly, and changes in lipid abundance have known associations with plant resistance in other systems, making these pathways good targets for further exploration (Biyiklioglu et al., 2018; Khajuria et al., 2013;

Lavergne et al., 2020; Zhu et al., 2012a) . Further examination of lignin content and composition in hollow, solid, and early-solid cultivars may reveal differences in stem architecture that influence the behavior of both adult and larval WSS. Additionally, more reliable methods for observing larval behavior in laboratory conditions will also allow for rigorous testing of the dietary requirements of WSS larvae to determine how lipid content or lignin subunit composition might affect larval growth and development.

Differentially expressed metabolites in the response of oat and wheat to infestation by WSS were also found which were associated with the salicylic acid signaling pathway, IAA degradation, and biosynthesis of 1,4-Benzoxazin-3-ones (Bxs). Some salicylic acid related compounds were found in higher abundance in oat samples of both cultivars, indicating that salicylic acid pathway defenses may be involved in the defense response to WSS in oat. Additional compounds related to plant defense that were identified in this study indicate the potential for defining the relationships between wheat, WSS and their parasitoids *B. cephi* and *B. lissogaster*. Bxs HDMBD-Glc and DIBOA-β-D-glucoside increased in all wheat cultivars after infestation with the exception of Conan, with oat expressing overall higher abundance of both compounds. Wheat also appears to respond to WSS infestation by inactivating IAA in stem tissues, while irreversible inactivation of IAA is dependent on cultivar. Further study of the effect of 1,4-benzoxazin-3-ones on sawfly development is necessary, especially since other studies of the sawfly system have identified changes in DIMBOA and related compounds in plants infested with WSS and the results have been highly variable (Biyiklioglu et al., 2018; Lavergne et al., 2020). There is evidence that both 1,4-Benzoxazin-3-ones and IAA pathway-related compounds not only have an effect on larval pests but can either positively or negatively

affect parasitoid populations so it is unclear what this means for braconid parasitoid populations in the WSS tritrophic system.

Ultimately, though solid stems do offer resistance to the plant, continued exploration of the tritrophic interaction between wheat, WSS and their parasitoids will help in mitigating negative effects on this system to maximize the success of control efforts. Additionally, increased abundance of metabolites in oat which are related to lignin biosynthesis and composition as well as increased abundance of several phospho- and galactolipids illustrate the need for further investigation of stem architecture at the molecular level as well as examination of the developmental and behavioral effects these metabolites have on WSS at all stages of development.

Acknowledgements

Funding for the Montana State Mass Spectrometry Facility used in this publication was made possible in part by the MJ Murdock Charitable Trust, the National Institute of General Medical Sciences of the National Institutes of Health under Award Numbers P20GM103474 and S10OD28650, and the MSU Office of Research and Economic Development. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This research was also supported by grants from the Montana Wheat and Barley Committee and by USDA National Institute of Food and Agriculture.

Figure 2.1. Principal component analysis of the oat and wheat dataset

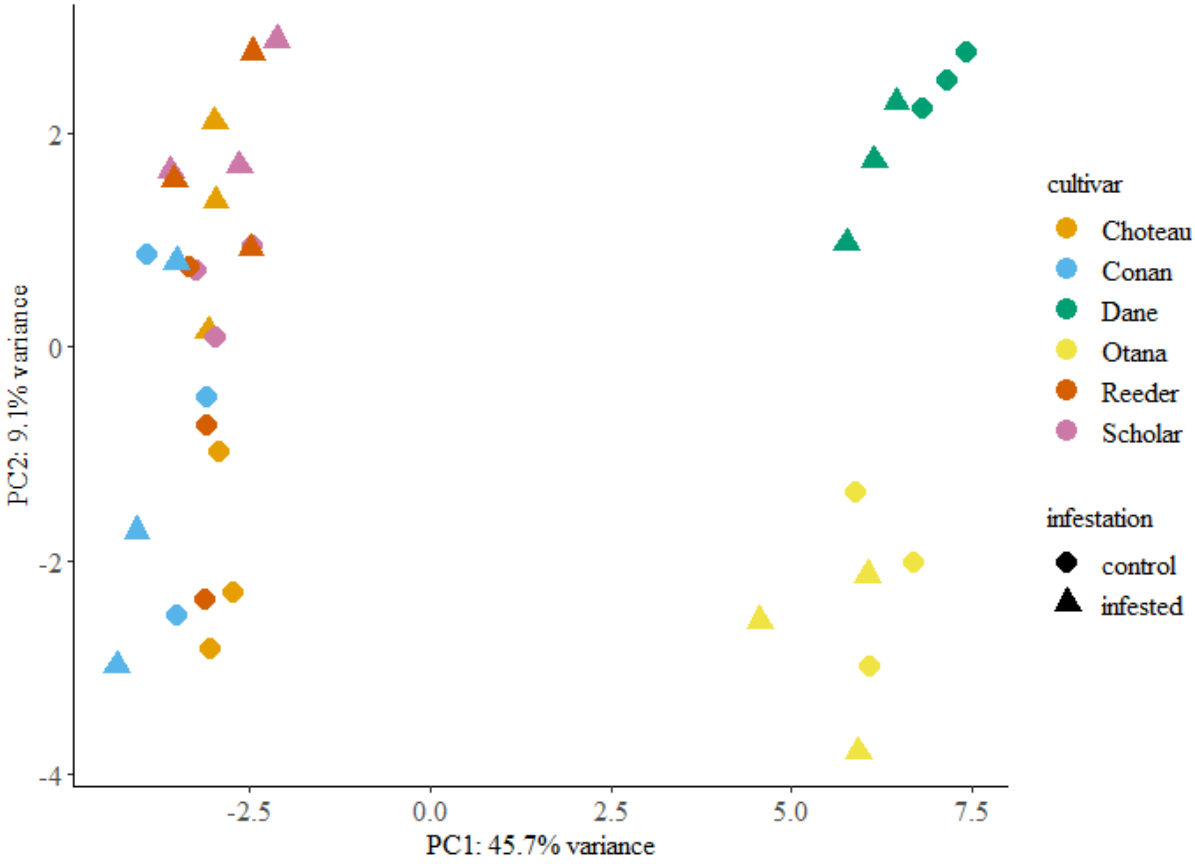


Figure 2.2. Pathway associations of significant compounds in the oat and wheat dataset

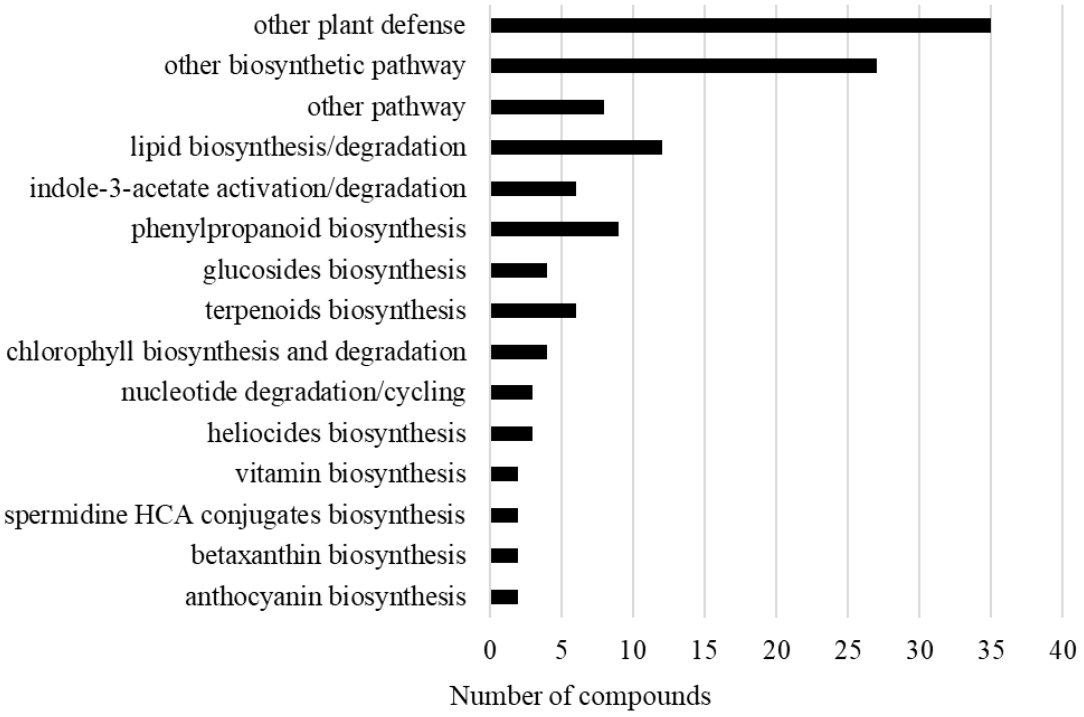


Figure 2.3. Classifications of significant metabolites from the oat and wheat dataset

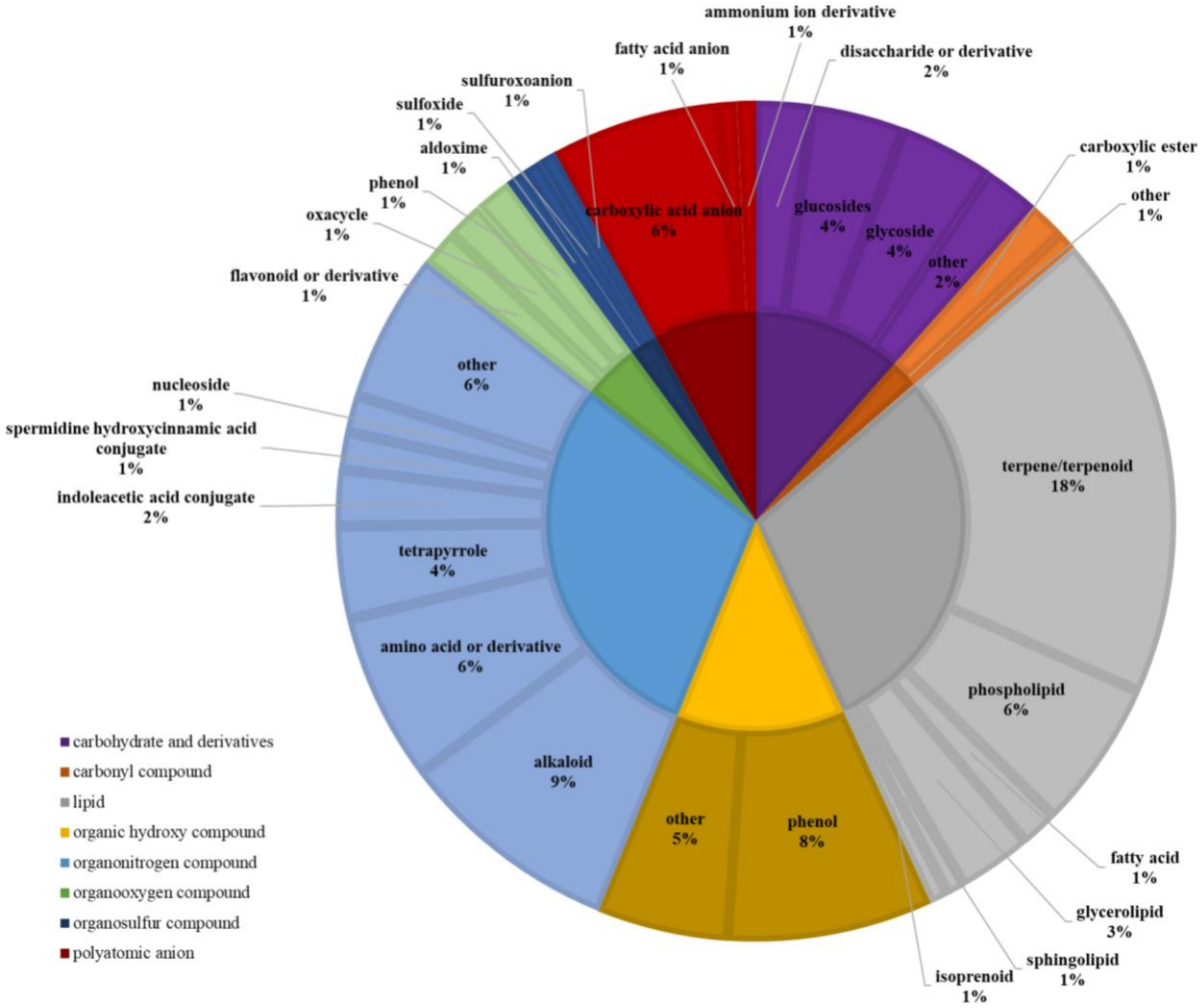


Figure 2.4. Abundance of hydroxycinnamate (HCA) esters in oat and wheat

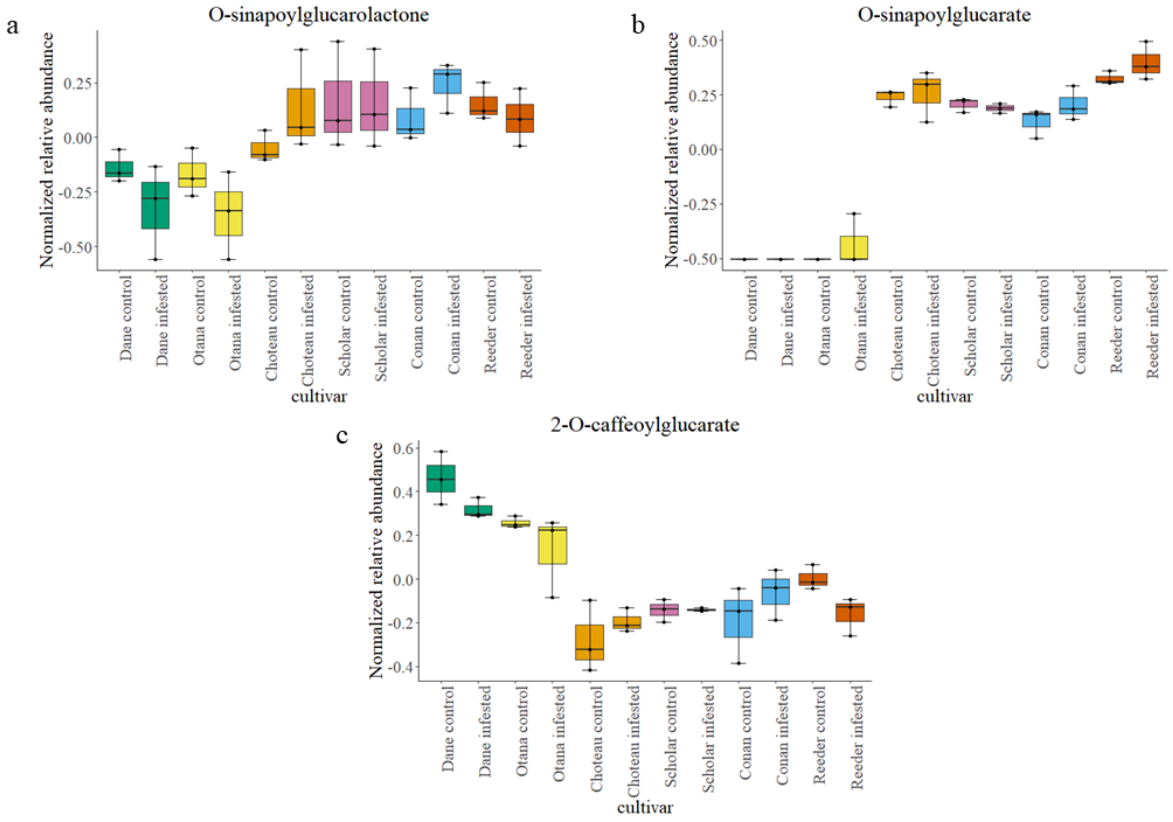


Figure 2.5. Abundance of suberin and lignin biosynthesis related metabolites

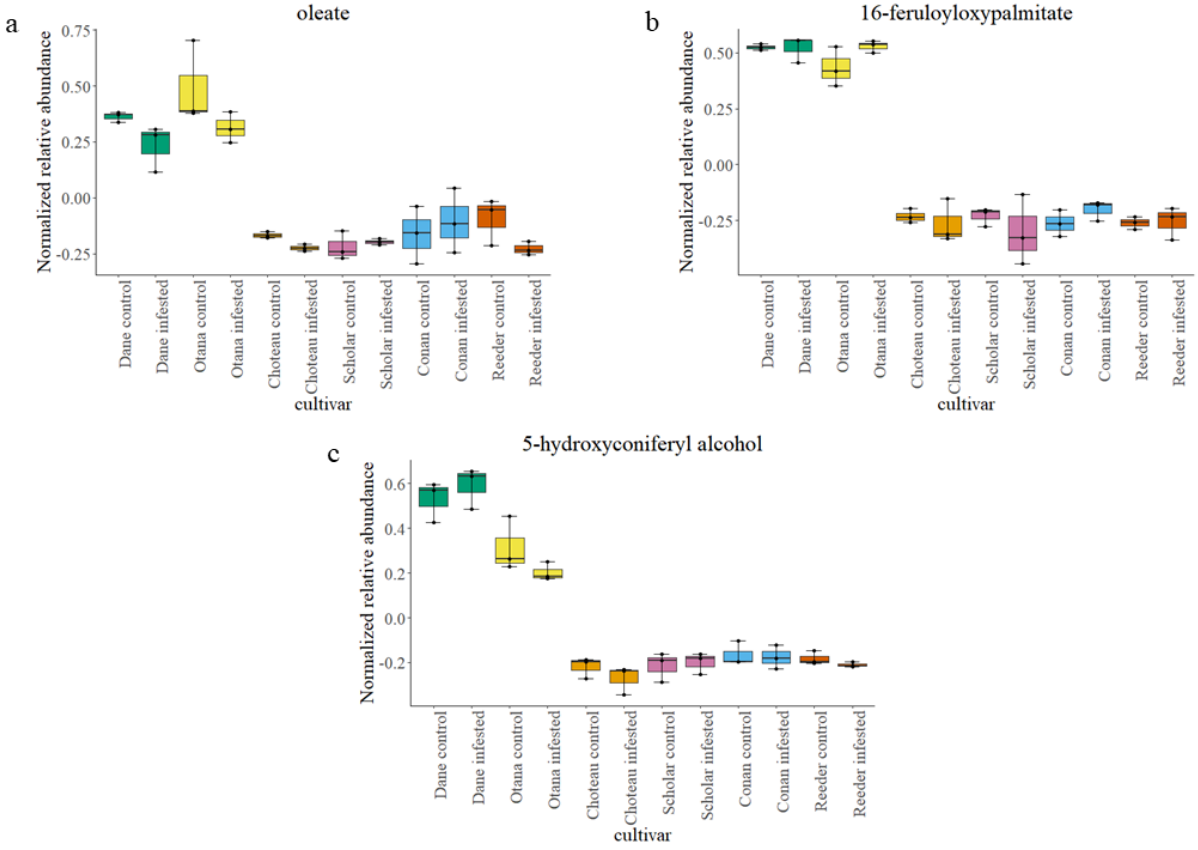
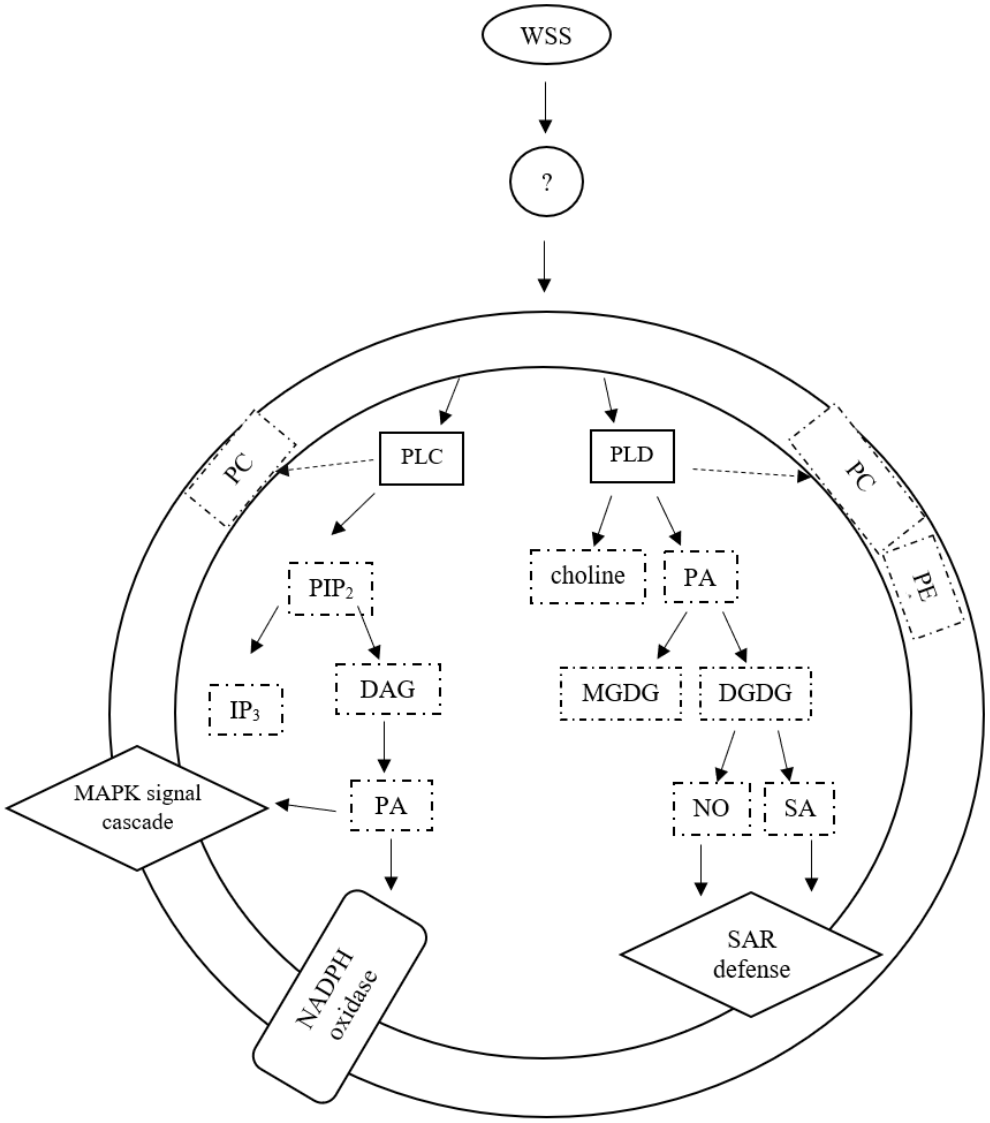


Figure 2.6. Phospho- and galactolipid signaling pathway



Putative pathway for plant cell phospho- and galactolipid response to WSS infestation. Squares indicate relevant enzymes, dashed boxes indicate molecules, dashed arrows indicate enzyme activity, dotted arrows indicate indirect activity, and parallelograms indicate defense pathways PC-phosphatidylcholine, PE-phosphatidylethanolamine, PLC-phospholipase C, PLD-phospholipase D, PIP2-phosphatidylinositol 4,5-bisphosphate, IP3- inositol 1,4,5-trisphosphate, DAG- diacylglycerol, PA-phosphatidic acid, P-phosphatidate, MGDG-monogalactosyldiacylglycerol, DGDG- digalactosyldiacylglycerol, SA-salicylic acid, NO-nitric oxide

Figure 2.7. Abundance of phospho- and galactolipids in oat and wheat

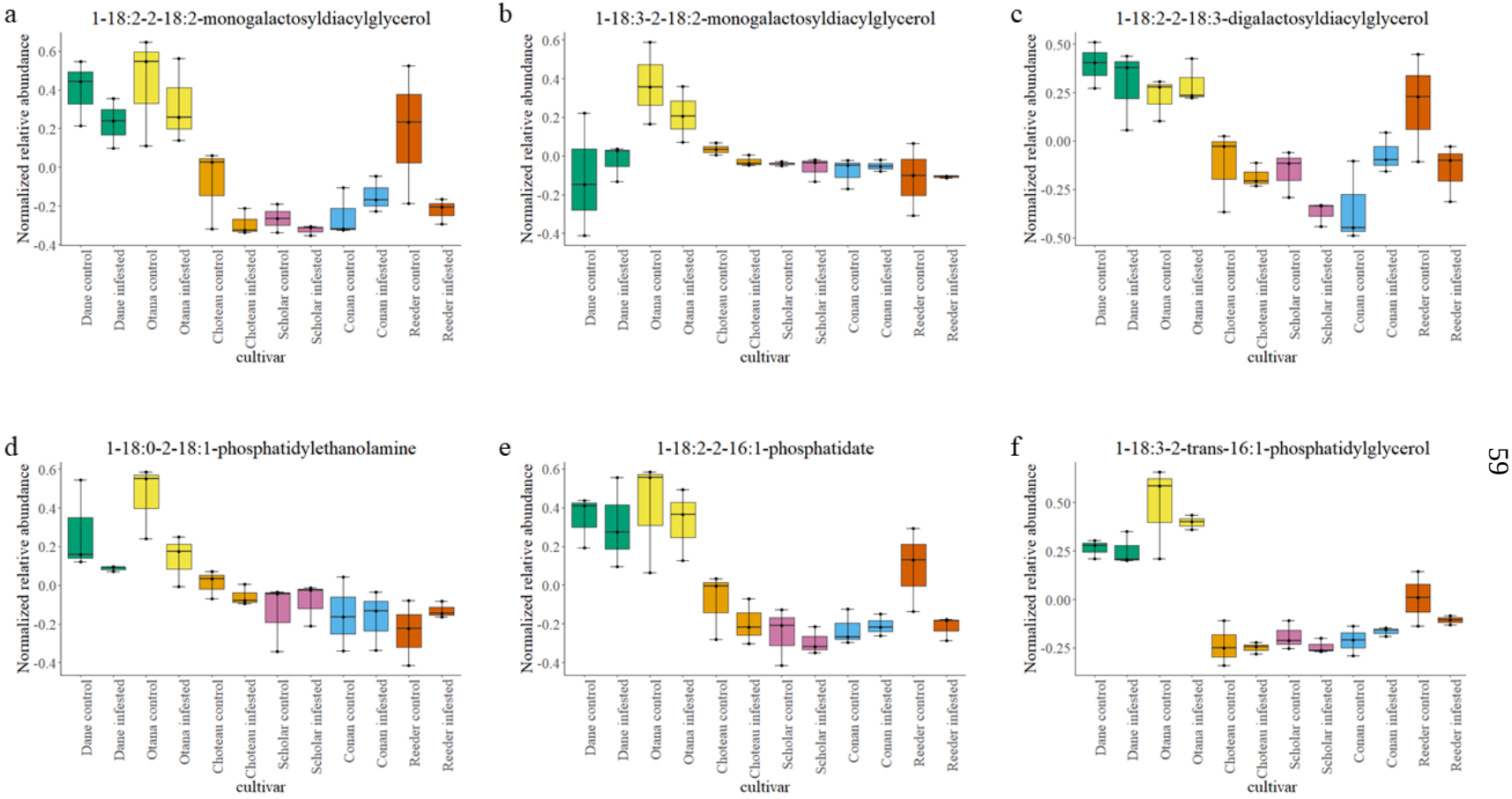


Figure 2.8. Abundance of salicylic acid related metabolites in oat and wheat

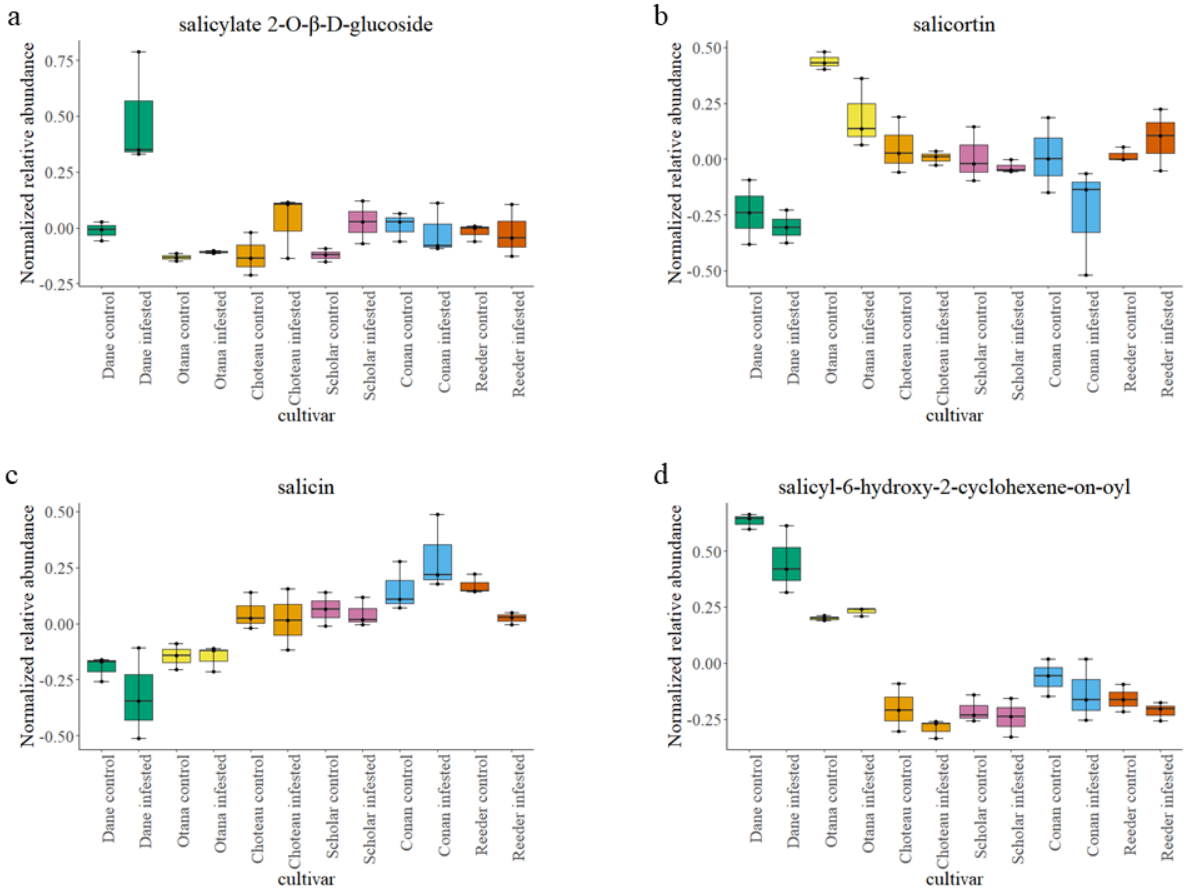


Figure 2.9. Abundance of metabolites associated with IAA degradation

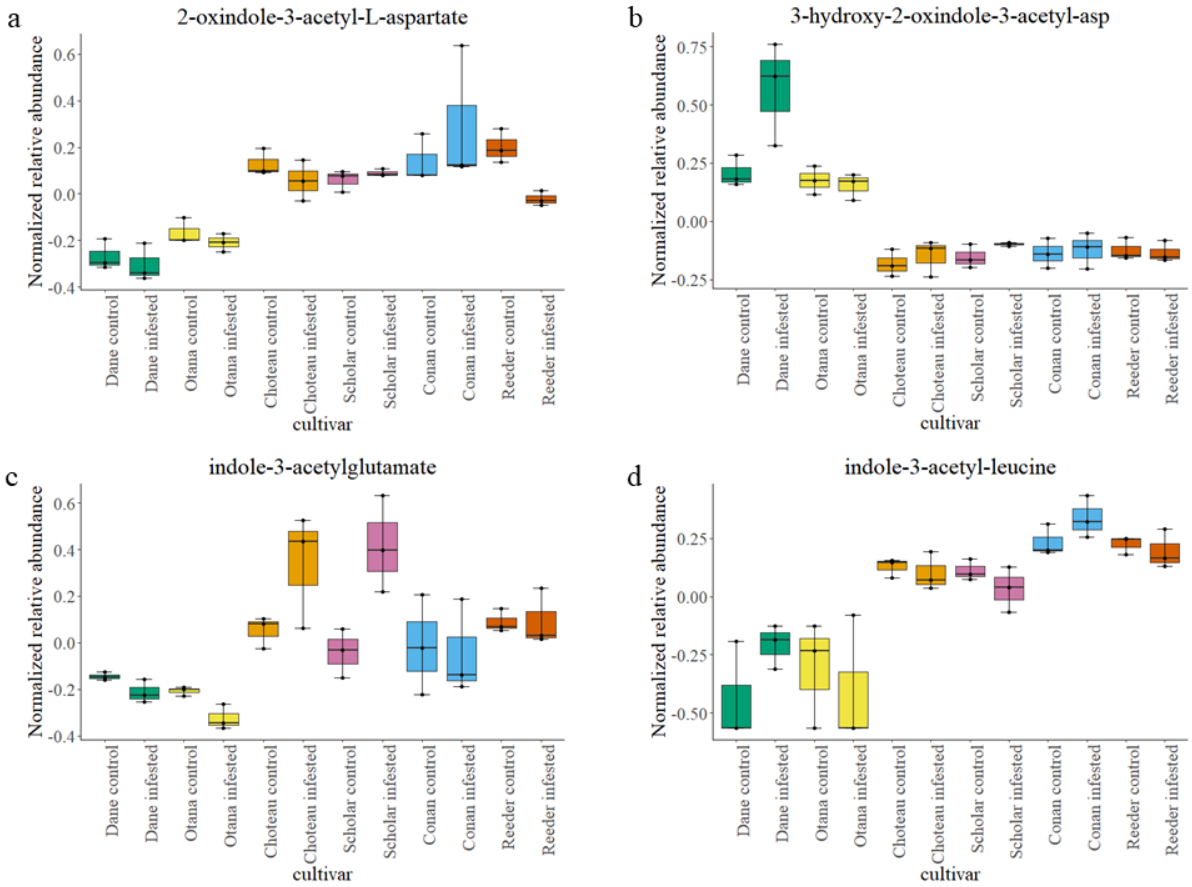


Figure 2.10. Principal component analysis of oat dataset

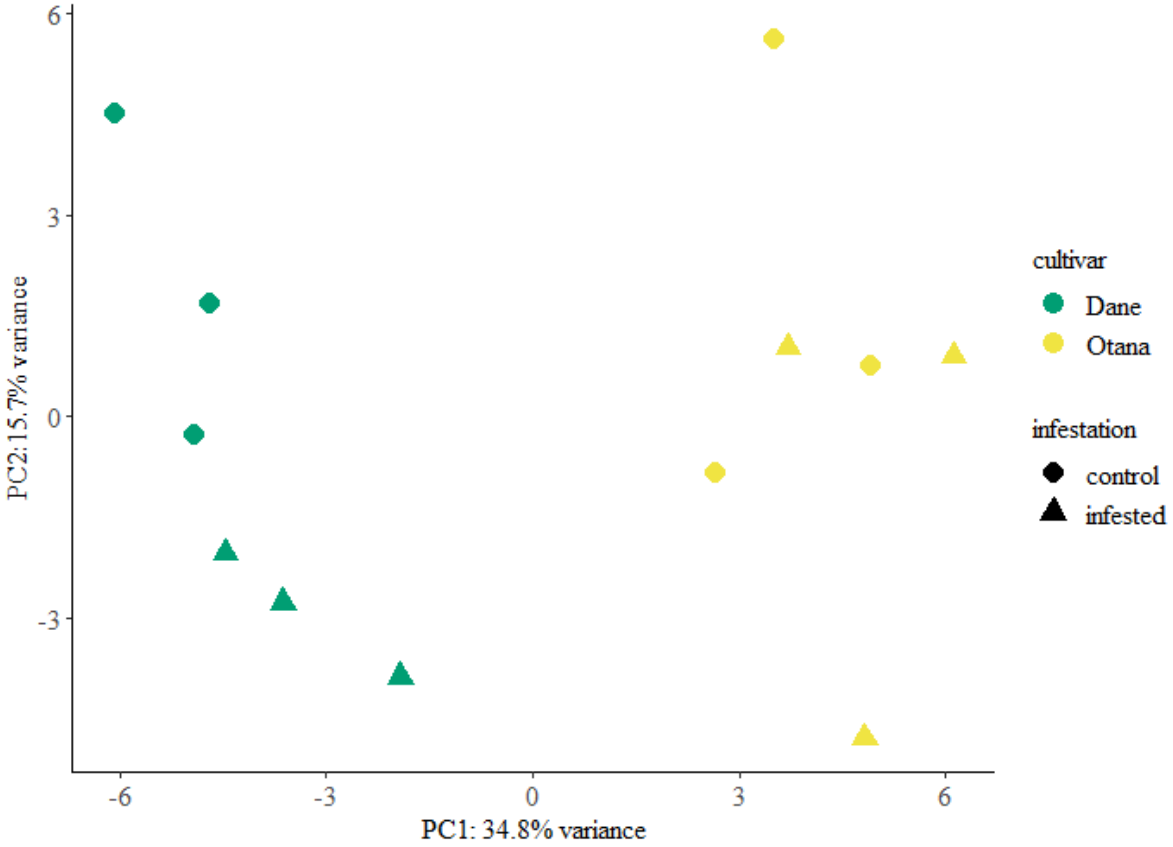


Figure 2.11. Pathway associations of significant compounds in the oat dataset

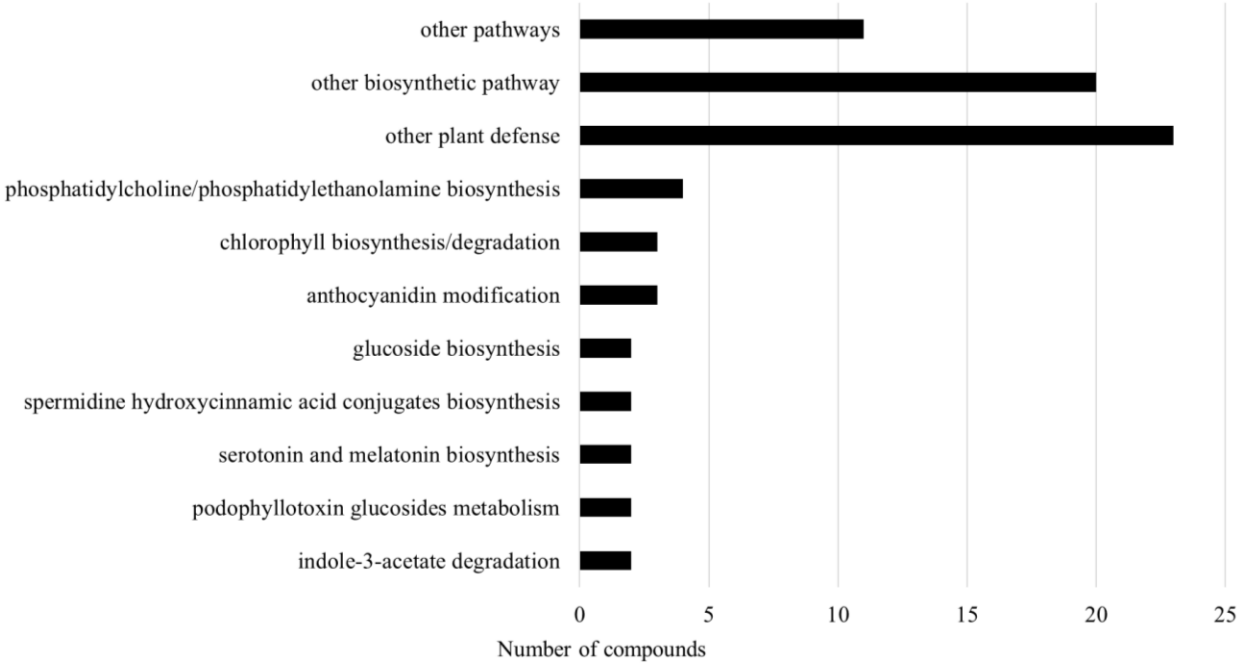


Figure 2.12. Classifications of significant metabolites from the oat dataset

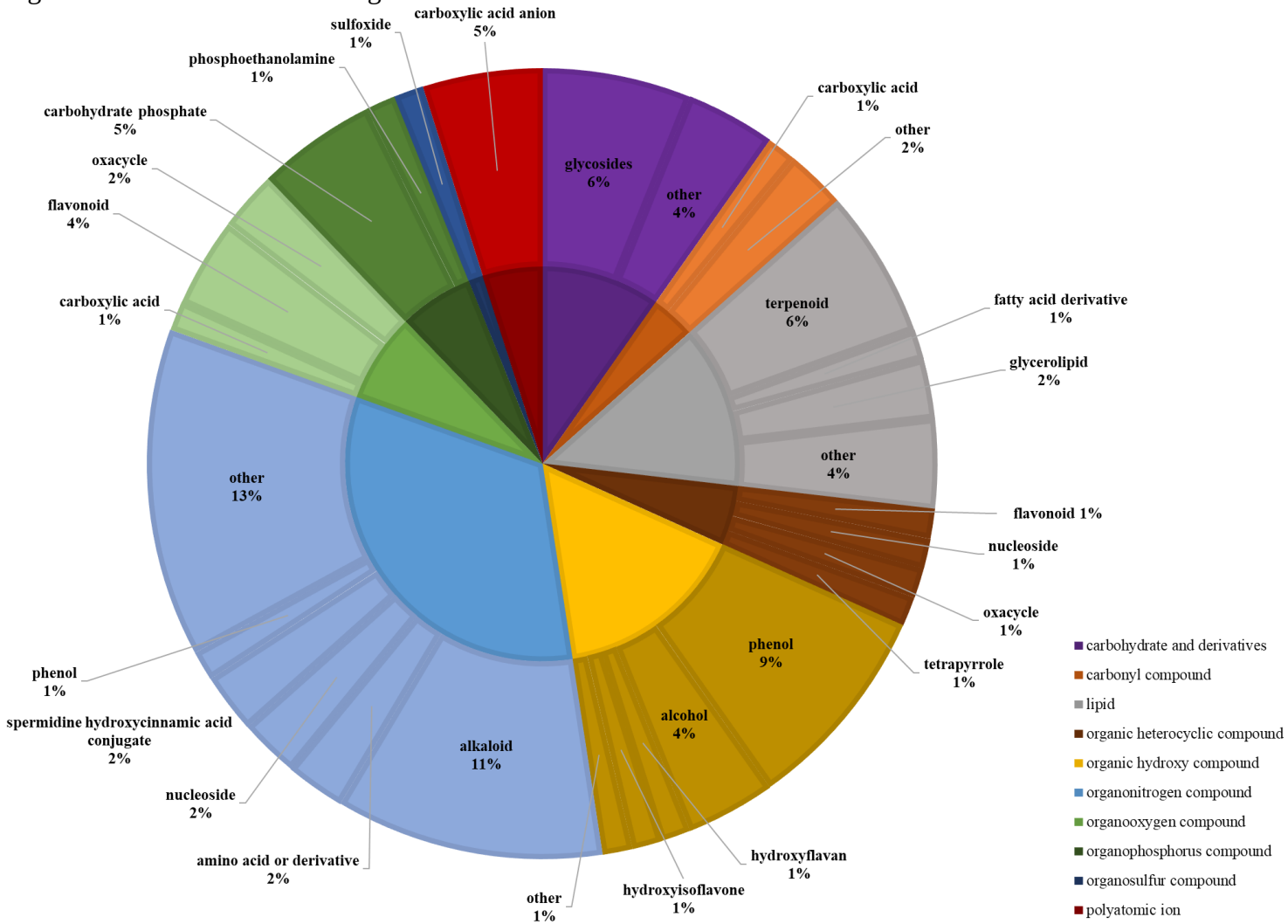


Figure 2.13. Principal component analysis of the wheat dataset

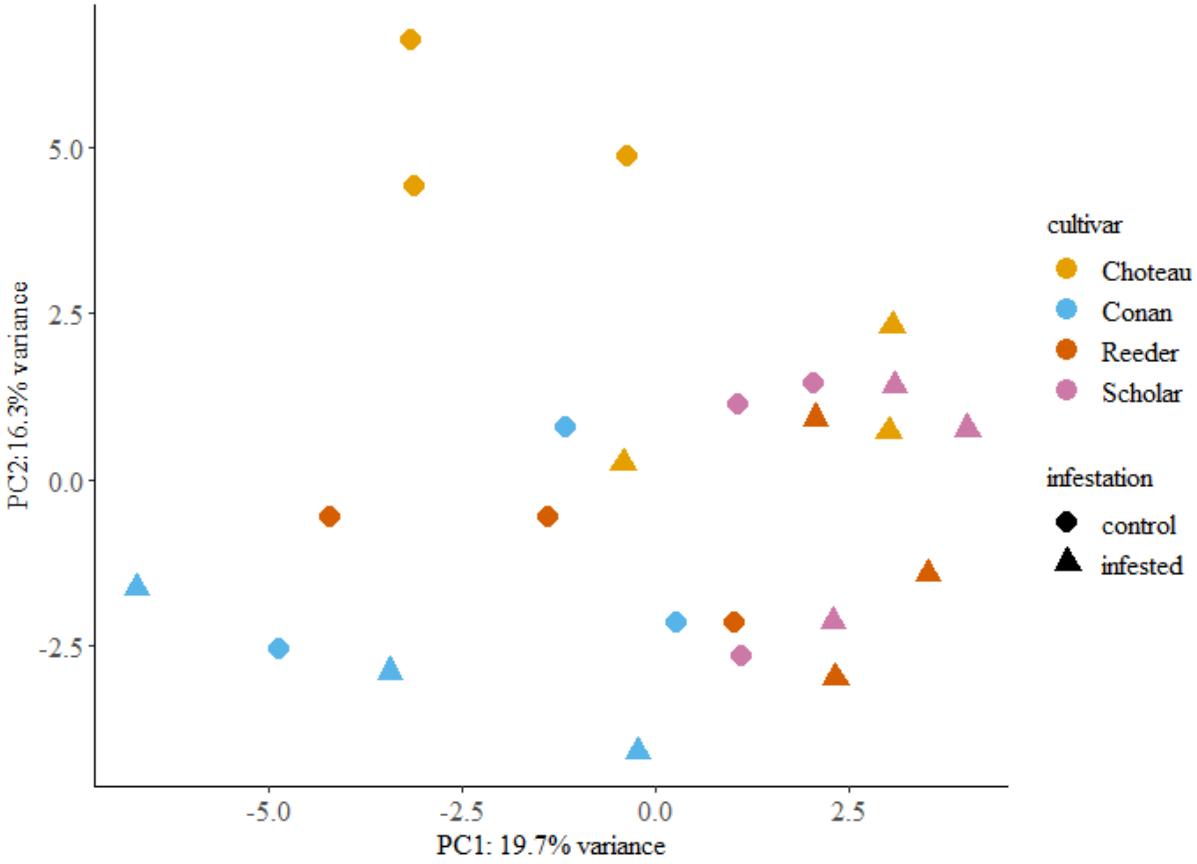


Figure 2.14. Pathway associations of significant compounds in the wheat dataset

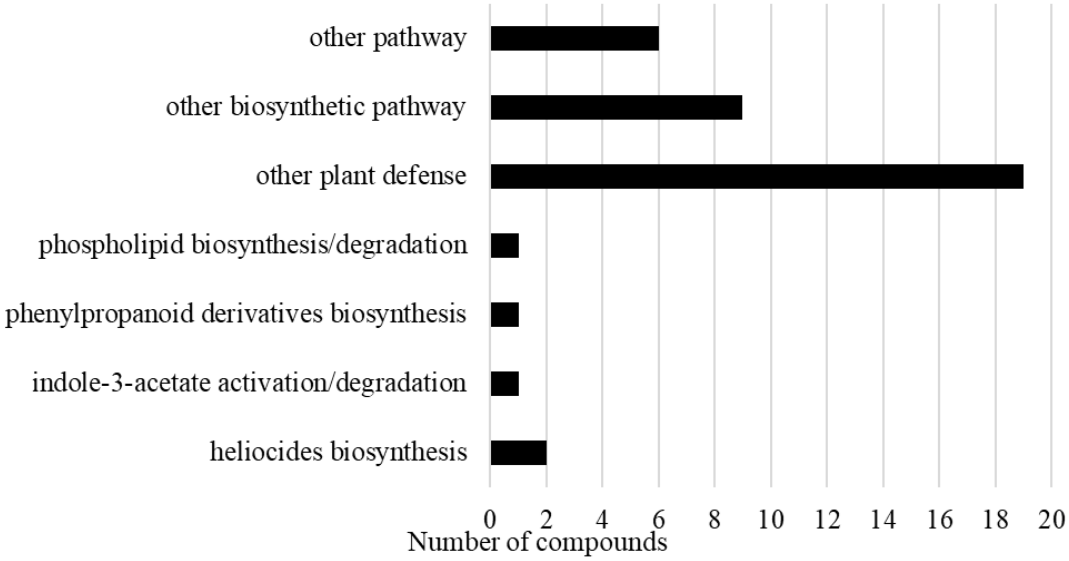


Figure 2.15. Classifications of significant metabolites from the wheat dataset

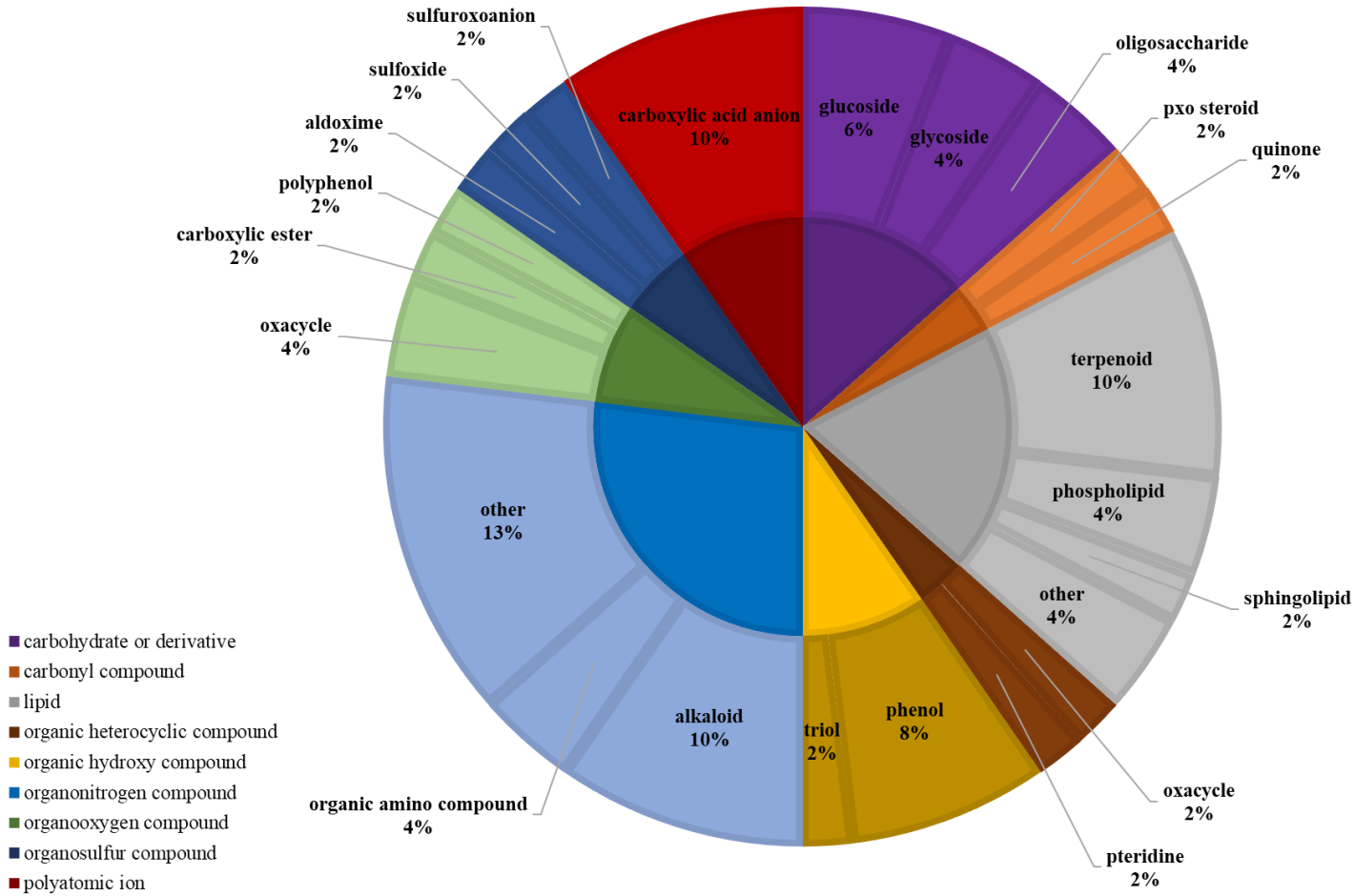


Figure 2.16. Abundance of DIMBOA related metabolites in oat and wheat

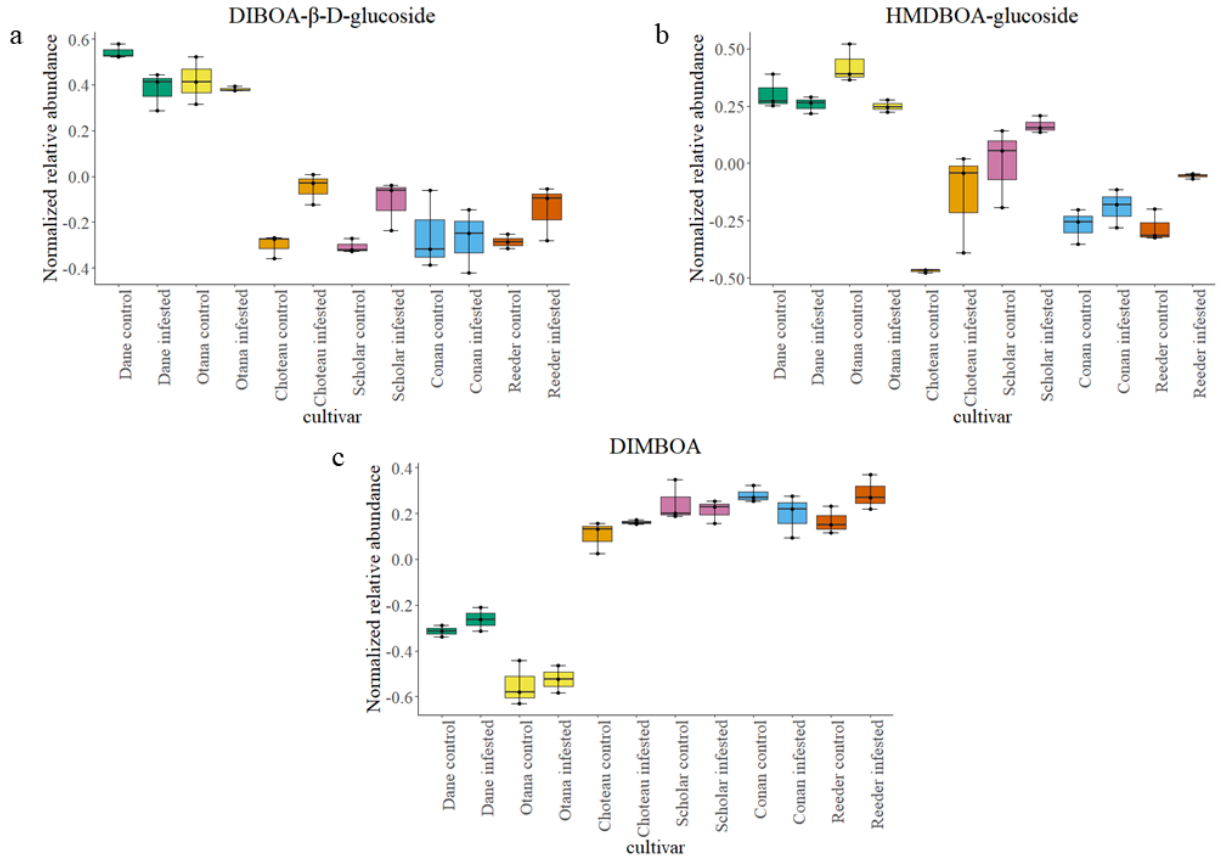


Table 2.1. Results from two-way ANOVA from oat and wheat dataset

Compound	m/z	R.T.	Formula	Status F val	Status adj p-val	Cultivar F val	Cultivar adj p-val	Interaction F val	Interaction adj p-val
1-(β-D- ribo furanosyl) nicotinamide	255.099	9.708	C11H15N2O5	0.160	0.877	6.159	0.001	0.743	0.769
1-18:0-2-18:1 phosphatidylethanolamine	746.556	19.787	C41H80N1O8P1	2.186	0.385	11.279	0.000	1.858	0.440
1-18:1-2-18:1 phosphatidate	700.496	24.492	C39H71O8P1	0.531	0.717	4.054	0.010	0.749	0.769
1-18:1-2-18:1 phosphatidylethanolamine	744.543	22.409	C41H78N1O8P1	5.890	0.167	7.006	0.001	1.942	0.417
1-18:2-2-16:1 phosphatidate	670.461	28.133	C37H65O8P1	2.897	0.319	17.059	0.000	0.736	0.769
1-18:2-2-18:2 digalactosyldiacylglycerol	940.593	24.377	C51H88O15	1.518	0.505	25.293	0.000	2.894	0.206

Table 2.1 continued

1-18:2-2-18:2 mono galactosyl diacylglycerol	778.545	27.431	C45H78O10	5.860	0.167	15.008	0.000	1.361	0.543
1-18:2-2-18:3 digalactosyl diacylglycerol	938.581	23.841	C51H86O15	1.370	0.522	15.248	0.000	2.728	0.232
1-18:3-2-18:2 mono galactosyl diacylglycerol	776.541	21.374	C45H76O10	0.184	0.866	6.998	0.001	0.577	0.835
1-18:3-2-trans-16:1 phosphatidyl glycerol	742.492	27.858	C40H70O10P1	1.376	0.522	49.646	0.000	0.511	0.860
1,2-dipalmitoyl phosphatidyl choline	734.562	24.832	C40H80N1O8P1	3.424	0.318	9.806	0.000	1.534	0.518
1-O-galloyl-β-D-glucose	332.082	13.765	C13H16O10	1.827	0.440	29.256	0.000	0.861	0.724
10β, 14β-dihydroxytaxa-4(20), 11-dien-5α-yl acetate	346.256	11.445	C22H34O3	0.660	0.689	6.088	0.001	1.548	0.518

Table 2.1 continued

10-deacetyl-2-debenzoyl baccatin III	440.198	7.100	C22H32O9	0.026	0.977	199.230	0.000	10.561	0.002
12-hydroxy dihydro chelirubine	379.097	7.603	C21H17N1O6	0.982	0.593	26.825	0.000	1.311	0.565
16-feruloyloxy palmitate	448.286	21.311	C26H39O6	0.161	0.877	179.050	0.000	1.102	0.616
16-methoxy tabersonine	367.205	9.737	C22H27N2O3	0.048	0.947	6.521	0.001	1.058	0.633
2-(3-benzoyl phenyl)-3,5,7 trihydroxy chromen-4-one	374.073	8.422	C22H12O6	3.662	0.300	81.555	0.000	0.639	0.815
2-omega-hydroxy-C22:0-LPA	510.338	28.332	C25H49O8P1	12.375	0.044	17.971	0.000	3.018	0.188
2,4-dihydroxy cinnamate	180.037	14.885	C9H8O4	4.597	0.215	42.909	0.000	1.660	0.482
2α,7β-dihydroxy taxusin	536.261	10.454	C28H40O10	1.488	0.509	47.402	0.000	0.301	0.950
2α-hydroxy taxusin	520.270	6.670	C28H40O9	0.450	0.733	16.179	0.000	1.676	0.482

Table 2.1 continued

2-amino-6-hydroxymethyl-7,7-dimethyl-7,8-dihydropteridine-4-one	223.105	4.210	C9H13N5O2	0.019	0.985	7.289	0.000	0.113	0.993
2-hydroxy phenylacetate	152.056	2.215	C8H7O3	0.245	0.826	13.747	0.000	0.580	0.835
2-O-caffeoyl glucarate	372.074	5.105	C15H14O11	0.990	0.593	29.577	0.000	2.014	0.396
2-oxindole-3-acetyl-L-aspartate	306.079	1.988	C14H12N2O6	0.793	0.633	19.803	0.000	2.014	0.396
2-oxoglutaramate	145.028	7.842	C5H6N1O4	1.828	0.440	29.728	0.000	0.582	0.835
24-epicathasterone-22-O-sulfate	512.314	11.470	C28H47O6S1	3.160	0.319	33.725	0.000	1.190	0.590
24-methylene cycloartanol	440.406	16.792	C31H52O1	2.172	0.385	12.838	0.000	0.524	0.854
3-(O-β-glucosyl)-2-oxindole-3-acetyl-asp	484.138	4.909	C20H22N2O12	5.579	0.169	5.640	0.001	3.258	0.152
3-hydroxy-2 oxindole-3 acetyl-asp	322.078	6.596	C14H12N2O7	6.390	0.164	41.774	0.000	4.278	0.086

Table 2.1 continued

3-hydroxyquinine	341.192	11.025	C20H25N2O3	0.017	0.985	7.628	0.000	1.839	0.440
(3E)- phyto chromobilin	584.280	11.484	C33H34N4O6	0.163	0.877	53.224	0.000	1.937	0.417
4,21- dehydro geissoschizine	351.181	7.076	C21H23N2O3	2.931	0.319	8.594	0.000	1.599	0.495
4-amino butanoate	104.069	2.657	C4H9N1O2	0.842	0.624	16.627	0.000	1.849	0.440
4-hydroxy-L-threonine	136.062	4.463	C4H9N1O4	0.081	0.918	45.453	0.000	1.387	0.530
4-hydroxy butanoate	104.051	1.171	C4H7O3	1.199	0.557	31.601	0.000	2.080	0.381
4-methylthio butyl hydroximate	165.025	8.423	C5H11N1O1S2	7.641	0.138	17.305	0.000	3.103	0.174
5-[[4-methoxy-3-(phenylmethoxy)phenyl]methyl]-2,4-pyrimidinediamine	336.164	8.348	C19H20N4O2	2.512	0.360	11.098	0.000	1.079	0.625
5,6- dihydro thymine	128.049	4.257	C5H8N2O2	2.745	0.335	17.395	0.000	1.867	0.440
5-hydroxy coniferyl alcohol	196.066	7.342	C10H12O4	0.906	0.607	150.360	0.000	1.189	0.590

Table 2.1 continued

6- hydroxy flavone	238.070	6.798	C15H10O3	1.515	0.505	37.831	0.000	0.700	0.786
6- methoxy podophyllo toxin	444.148	7.789	C23H24O9	0.409	0.750	59.291	0.000	4.302	0.086
6-methyl sulfinylhexyl glucosinolate	369.127	8.983	C14H27N1O6S2	0.018	0.985	48.689	0.000	0.920	0.691
7,8- dihydro berberine	337.135	5.776	C20H19N1O4	3.049	0.319	23.986	0.000	0.242	0.964
7-deoxy loganetate	198.083	7.379	C10H13O4	9.661	0.077	4.720	0.005	4.052	0.100
7-hydroxy-4’ 5’-dimethoxy isoflavone	298.075	5.339	C17H14O5	3.350	0.319	107.950	0.000	3.733	0.109
7-hydroxy flavone	238.055	1.713	C15H10O3	0.471	0.733	6.955	0.001	6.072	0.032
7-methylthio heptyl glucosinolate	463.088	4.420	C15H28N1O9S3	0.559	0.705	49.000	0.000	1.797	0.451
8-hydroxy-(+)-δ-cadinene	220.176	9.996	C15H24O1	0.013	0.986	11.954	0.000	1.188	0.590
8-methylthio octanaldoxime	189.110	8.799	C9H19N1O1S1	0.000	0.991	17.855	0.000	1.823	0.442

Table 2.1 continued

9-methylthio nonyl hydroximate	221.089	2.161	C9H19N1O1S2	0.001	0.991	5.734	0.001	2.316	0.326
(9Z, 11E, 14Z, 13S)-hydro peroxylocta deca-9, 11, 14-trienoate	310.206	12.916	C18H29O4	0.001	0.991	8.275	0.000	1.008	0.653
(9Z, 12E) octadeca dienoate	280.233	21.735	C18H31O2	0.810	0.633	3.208	0.027	0.798	0.759
adenosine 5'-phosphor amidate	346.079	10.714	C10H14N6O6P1	0.563	0.705	64.620	0.000	1.459	0.524
adonirubin	580.378	10.226	C40H52O3	0.014	0.986	79.611	0.000	1.957	0.417
ajmaline	326.201	4.944	C20H26N2O2	0.082	0.918	35.942	0.000	1.119	0.613
alizarin	240.037	4.060	C14H8O4	1.832	0.440	26.044	0.000	0.265	0.962
allantoate	176.053	1.700	C4H7N4O4	0.126	0.880	16.460	0.000	0.169	0.981
α-ketol	312.222	11.899	C18H31O4	1.230	0.551	15.279	0.000	0.701	0.786
α-tocotrienol	424.341	26.060	C29H44O2	3.215	0.319	17.650	0.000	1.391	0.530
benzoyl-β-D glucopyranose	284.090	14.237	C13H16O7	25.806	0.003	20.943	0.000	19.665	0.000
β-D-gluco pyranosyl abscisate	426.193	17.135	C21H30O9	2.213	0.385	50.840	0.000	0.933	0.686
β-primeverose	312.107	1.314	C11H20O10	0.477	0.733	58.156	0.000	1.765	0.451
bixin aldehyde	348.203	11.608	C24H28O2	0.236	0.826	20.669	0.000	1.109	0.616

Table 2.1 continued

bixin dimethyl ester	408.240	13.886	C26H32O4	0.900	0.607	23.447	0.000	2.050	0.393
bornyl diphosphate	314.071	9.266	C10H17O7P2	10.260	0.077	38.219	0.000	1.166	0.595
calcitriol	416.320	15.846	C27H44O3	1.857	0.440	30.966	0.000	1.630	0.486
capsorubin	600.415	25.045	C40H56O4	3.000	0.319	8.361	0.000	1.230	0.582
cocaine	304.163	4.862	C17H22N1O4	4.553	0.215	15.866	0.000	1.638	0.486
crepenynate	278.217	16.577	C18H29O2	0.066	0.925	23.079	0.000	0.781	0.759
curcumin	368.118	16.744	C21H20O6	2.407	0.367	59.980	0.000	2.887	0.206
curcumin mono glucoside	530.163	13.289	C27H30O11	0.001	0.991	62.278	0.000	1.268	0.568
Cyanidin 3-(p-coumaroyl) glucoside	595.151	12.012	C30H26O13	0.001	0.991	20.790	0.000	3.297	0.150
cyclo eucalenone	424.375	27.376	C30H48O1	5.755	0.167	20.013	0.000	0.916	0.691
D-nopaline	306.145	4.560	C11H19N4O6	1.109	0.572	23.652	0.000	2.127	0.377
dehydro abietadiene	270.229	21.546	C20H30	0.510	0.725	5.782	0.001	0.476	0.869
dehydro spermidine	146.164	3.246	C7H20N3	2.512	0.360	3.976	0.011	2.703	0.237
δ-cadinene	204.181	12.376	C15H24	5.751	0.167	33.261	0.000	2.385	0.316
deoxy pumiloside	496.195	7.708	C26H28N2O8	9.682	0.077	53.482	0.000	1.161	0.595
DIBOA-β-D glucoside	343.100	6.262	C14H17N1O9	4.057	0.250	78.170	0.000	4.364	0.086
dihydro curcumin	370.133	9.022	C21H22O6	8.459	0.114	5.553	0.003	3.517	0.126

Table 2.1 continued

dihydro sorgoleone	344.243	11.363	C22H32O3	0.000	0.991	3.124	0.031	1.255	0.568
dimeric urushiol peroxide	658.430	26.767	C42H58O6	7.087	0.147	14.374	0.000	2.856	0.211
dipalmitoyl phosphatidate	648.484	27.336	C35H67O8P1	5.740	0.167	8.480	0.000	2.369	0.318
dopaxanthin	390.114	8.887	C18H15N2O8	0.139	0.880	3.926	0.012	2.185	0.356
dopaxanthin quinone	388.102	11.045	C18H13N2O8	0.561	0.705	111.230	0.000	0.434	0.891
(E)-2-methoxy carbonyl methyl butenedioate	188.030	10.029	C7H6O6	0.767	0.639	26.430	0.000	0.551	0.851
ε-carotene	536.424	23.239	C40H56	3.302	0.319	16.801	0.000	0.953	0.685
eupatolin	492.133	10.131	C23H24O12	0.400	0.751	80.916	0.000	0.479	0.869
feruloyl agmatine	307.173	6.318	C15H23N4O3	4.183	0.248	3.864	0.012	2.099	0.381
geranyl geranyl chlorophyll b	900.459	11.756	C55H64N4O6Mg1	5.210	0.183	47.111	0.000	0.398	0.904
ginsenoside Rg1	800.508	22.372	C42H72O14	2.465	0.363	13.519	0.000	0.906	0.696
glutathione disulfide	614.184	10.862	C20H30N6O12S2	1.170	0.558	6.490	0.001	0.530	0.854
gossypol-6,6’ dimethyl ether	546.228	10.318	C32H34O8	0.002	0.991	12.456	0.000	0.059	0.997
heliocide B2	424.214	10.167	C26H31O5	0.002	0.991	6.880	0.001	0.593	0.835
heliocide H4	410.198	7.529	C25H29O5	0.051	0.946	82.554	0.000	3.958	0.100
hemigossypol	260.107	8.859	C15H16O4	27.071	0.003	52.668	0.000	2.228	0.354

Table 2.1 continued

indole-3-acetyl-glu	304.115	14.885	C15H14N2O5	3.633	0.300	11.538	0.000	4.123	0.100
indole-3-acetyl-iso	288.142	6.373	C16H19N2O3	12.219	0.044	122.830	0.000	1.081	0.625
indole-3-acetyl leu	288.152	1.666	C16H19N2O3	0.143	0.880	23.736	0.000	1.270	0.568
indole-3-acetyl-phenylalanine	322.131	8.528	C19H17N2O3	1.991	0.422	10.551	0.000	1.012	0.653
indole-3-acetyl proline	272.110	6.791	C15H15N2O3	5.373	0.176	9.874	0.000	0.865	0.724
Inosine	268.085	8.749	C10H12N4O5	0.440	0.733	14.632	0.000	1.456	0.524
Isoliquiritigenin 4'-glucoside	418.115	10.965	C21H22O9	6.548	0.162	36.987	0.000	0.217	0.971
kaempferide triglycoside	770.215	7.823	C34H42O20	3.074	0.319	92.780	0.000	5.258	0.050
kauralexin A2	334.217	6.849	C20H28O4	0.619	0.702	10.993	0.000	1.275	0.568
kauralexin A3	334.205	15.542	C20H28O4	1.756	0.455	14.047	0.000	1.263	0.568
L-cysteine	122.026	5.999	C3H7N1O2S1	2.957	0.319	9.173	0.000	3.847	0.109
L-hyoscyamine	290.168	1.909	C17H24N1O3	0.135	0.880	22.132	0.000	0.594	0.835
larreatricin	284.150	4.225	C18H20O3	0.269	0.812	3.255	0.026	0.428	0.892
lathyrine	183.085	12.905	C7H10N4O2	0.346	0.770	11.338	0.000	1.180	0.590
leucocyanidin	306.074	6.382	C15H14O7	0.004	0.991	50.744	0.000	1.207	0.590
lupan-3-β,20-diol	444.407	28.534	C30H52O2	2.383	0.368	14.128	0.000	0.681	0.793
lupinate	307.148	16.226	C13H18N6O3	0.451	0.733	6.357	0.001	1.460	0.524
Luteolin-7-O β-D-glucuronide	462.072	1.660	C21H17O12	7.899	0.133	48.160	0.000	2.667	0.241

Table 2.1 continued

N-acetyl farnesyl cysteine	367.228	6.663	C20H32N1O3S1	6.998	0.147	13.134	0.000	2.122	0.377
N-glucosyl nicotinate	286.089	3.756	C12H16N1O7	1.125	0.570	10.833	0.000	0.785	0.759
O-β-D xylosylzeatin	351.145	5.127	C15H21N5O5	0.352	0.770	128.440	0.000	1.028	0.652
O-sinapoyl glucarate	416.098	1.697	C17H18O12	2.909	0.319	207.550	0.000	0.629	0.818
O-sinapoyl glucarolactone	398.074	5.948	C17H18O11	0.077	0.918	8.315	0.000	1.427	0.527
oleate	282.249	20.714	C18H33O2	5.001	0.194	51.796	0.000	1.510	0.521
oxindole-3-acetyl-aspartate-N-β-glucose	468.131	14.410	C20H22N2O11	2.849	0.320	5.281	0.003	1.423	0.527
palmatine	352.161	6.187	C21H22N1O4	0.003	0.991	39.790	0.000	1.701	0.474
palustradiene	272.256	17.914	C20H32	1.577	0.497	3.064	0.033	1.408	0.530
pheophytin a	870.558	27.087	C55H74N4O5	0.604	0.705	39.184	0.000	1.258	0.568
phlorizin	436.138	6.205	C21H23O10	0.645	0.694	36.548	0.000	6.015	0.032
phyto sphingosine	318.297	12.145	C18H40N1O3	2.346	0.368	4.040	0.010	0.658	0.804
protoporphyrinogen IX	568.301	6.076	C34H38N4O4	0.378	0.762	5.989	0.001	1.384	0.530
pseudo baptigenin	282.045	1.096	C16H10O5	1.000	0.593	61.791	0.000	1.331	0.559
pyridoxal	167.052	9.997	C8H9N1O3	0.003	0.991	26.859	0.000	0.753	0.769

Table 2.1 continued

quercetin-3-rhamnoside-7-rhamnoside	596.164	7.802	C27H31O15	0.306	0.796	145.090	0.000	4.754	0.073
quinidine	325.198	17.940	C20H25N2O2	0.013	0.986	3.294	0.025	0.744	0.769
raucaffrinoline	352.183	5.829	C21H24N2O3	4.624	0.215	31.535	0.000	5.005	0.060
red chlorophyll catabolite	628.308	8.992	C35H38N4O7	1.634	0.484	24.628	0.000	0.695	0.786
rotenolone	410.146	6.425	C23H22O7	0.005	0.991	10.430	0.000	0.538	0.854
(S)-N- methyl canadine	354.173	7.432	C21H24N1O4	1.415	0.522	44.677	0.000	1.523	0.518
(S)-tetrahydro columbamine	341.157	5.803	C20H23N1O4	0.088	0.918	50.278	0.000	1.546	0.518
salicin	286.100	1.850	C13H18O7	0.791	0.633	16.933	0.000	1.489	0.521
salicin-6-phosphate	366.076	4.631	C13H17O10P1	1.832	0.440	13.968	0.000	1.184	0.590
salicyl-6-hydroxy-2-cyclohexene-on-oyl	262.089	4.016	C14H14O5	5.899	0.167	88.403	0.000	1.124	0.613
salicylate-2-O-β-D glucoside	300.091	6.021	C13H15O8	13.354	0.040	7.767	0.000	4.960	0.060
secoisolariciresinol	362.171	6.756	C20H26O6	0.076	0.918	2.943	0.038	0.492	0.866
secoisolariciresinol mono glucoside	524.225	4.988	C26H36O11	7.428	0.141	77.210	0.000	3.930	0.105
secologanin	388.147	10.957	C17H24O10	3.195	0.319	24.501	0.000	0.402	0.904

Table 2.1 continued

serotonin	177.100	2.548	C10H13N2O1	13.649	0.040	3.022	0.035	3.572	0.126
sterculate	294.248	25.693	C19H33O2	9.710	0.077	19.537	0.000	0.961	0.683
steviol monoside	480.276	11.955	C26H40O8	6.809	0.150	21.949	0.000	1.915	0.423
Stigmasterol-3-O-β-D glucoside	574.410	11.909	C35H58O6	1.029	0.593	7.720	0.000	1.730	0.461
tetrahydro geranyl geranyl PP	454.233	10.652	C20H37O7P2	0.480	0.733	62.001	0.000	0.980	0.672
tetrahydro pteroyl-tri-L glutamate	703.247	8.372	C29H33N9O12	2.325	0.368	40.575	0.000	8.812	0.004
tricoumaroyl spermidine	583.265	8.862	C34H37N3O6	0.010	0.989	28.460	0.000	0.832	0.740
tuberonic acid glucoside	388.162	19.443	C18H27O9	1.377	0.522	8.924	0.000	2.205	0.355
vicianose	312.103	6.271	C11H20O10	3.384	0.318	3.521	0.019	0.945	0.686
vitexin-2”-O-β-D-glucoside	594.149	12.039	C27H29O15	5.913	0.167	46.568	0.000	9.600	0.003
vomilenine	350.173	14.881	C21H22N2O3	0.092	0.918	7.341	0.000	1.428	0.527
xanthine	152.030	1.688	C5H4N4O2	11.749	0.044	2.895	0.040	3.449	0.126
zealexin A3	250.160	5.850	C15H21O3	0.935	0.607	12.777	0.000	1.661	0.482
zealexin B1	232.152	7.117	C15H19O2	0.592	0.705	4.179	0.009	0.855	0.724

Table 2.2. Results from two-way ANOVA oat dataset

Compound	m/z	R.T.	Formula	Status F val	Status adj p-val	Cultivar F val	Cultivar adj p-val	Interaction F val	Interaction adj p-val
1-(β-D ribo furanosyl) nicotinamide	255.099	9.708	C11H15N2O5	3.775	0.345	35.022	0.002	1.147	0.928
1-18:2-2-18: 2-digalactosyl diacylglycerol	940.593	24.377	C51H88O15	2.451	0.473	11.765	0.021	0.040	0.993
1-18:3-2-18: 2-monogalactosyl diacylglycerol	776.541	21.374	C45H76O10	9.061	0.170	73.523	0.000	3.712	0.700
1,4-β-D-glucan	666.225	13.890	C24H42O21	1.222	0.572	9.690	0.030	0.007	0.993
1,4-naphtho quinone	158.039	1.224	C10H6O2	26.582	0.043	36.408	0.001	23.025	0.050
1,5,7-trihydroxy-6,8-dimethoxy anthraquinone	316.053	1.988	C16H11O7	106.86	0.001	202.880	0.000	86.192	0.003
10-deacetyl-2 debenzoyl baccatin III	440.198	7.100	C22H32O9	1.974	0.493	8.758	0.037	0.042	0.993
12-hydroxy dihydro chelirubine	379.097	7.603	C21H17N1O6	5.854	0.269	79.387	0.000	2.018	0.900

Table 2.2 continued

2,5-dihydroxy benzoate 5-O-β-D glucoside	316.089	2.559	C13H15O9	2.329	0.477	19.208	0.006	0.000	0.999
2-C-methyl-D-erythritol-2,4-cyclo diphosphate	277.996	1.672	C5H10O9P2	4.066	0.327	8.649	0.037	0.014	0.993
2-deoxy-α-D-ribose 1 phosphate	214.030	6.073	C5H9O7P1	16.114	0.114	134.510	0.000	0.451	0.928
2-hydroxy succinamate	133.047	5.397	C4H6N1O4	1.750	0.493	40.664	0.001	0.229	0.953
2-methyl-3-hydroxy butyryl CoA	867.179	7.131	C26H40N7O18P3S1	1.145	0.575	43.528	0.001	0.017	0.993
2-O-caffeoyl glucarate	372.074	5.105	C15H14O11	0.083	0.901	18.135	0.008	0.489	0.928
26- hydroxy brassinolide	496.338	17.263	C28H48O7	0.098	0.901	15.299	0.011	4.541	0.680
(2Z,6Z)-farnesyl diphosphate	382.122	8.268	C15H25O7P2	0.021	0.939	9.319	0.034	1.483	0.928

Table 2.2 continued

3,5-dimethoxy phenol	154.060	7.748	C8H10O3	0.081	0.901	8.808	0.037	1.039	0.928
3-epihydroxy-2'-deoxy mugineate	321.133	6.911	C12H18N2O8	4.089	0.327	54.453	0.001	1.387	0.928
3-hydroxy-2-oxindole-3-acetyl-asp	322.078	6.596	C14H12N2O7	2.125	0.481	25.967	0.003	2.140	0.900
4-methyl umbelliferone 6'-O-malonyl glucoside	424.089	1.690	C19H19O11	0.916	0.592	19.855	0.006	1.328	0.928
5'-demethoxy -6-methoxy podophyllo toxin	414.141	5.099	C22H22O8	10.392	0.160	25.146	0.003	11.640	0.225
5,7-dihydroxy-2-methyl chromone	192.049	1.746	C10H8O4	0.051	0.910	62.536	0.000	0.025	0.993
6-hydroxy allocryptopine	385.159	13.551	C21H23N1O6	0.034	0.924	34.120	0.002	0.142	0.982
6-hydroxy flavone	238.070	6.798	C15H10O3	0.425	0.744	43.918	0.001	0.502	0.928
6-methoxy mellein	208.076	8.765	C11H12O4	0.810	0.620	8.747	0.037	0.833	0.928

Table 2.2 continued

6-methoxy podophyllo toxin	444.148	7.789	C23H24O9	0.000	0.995	36.194	0.001	0.720	0.928
6-methyl sulfinylhexyl glucosinolate	465.084	4.441	C14H26N1O10S3	10.477	0.160	219.320	0.000	0.003	0.993
7,8-dihydro berberine	337.135	5.776	C20H19N1O4	2.627	0.450	13.824	0.015	0.301	0.953
7-hydroxy flavone	238.055	1.713	C15H10O3	11.939	0.154	33.610	0.002	5.879	0.500
7-methylthio heptyldesulfo glucosinolate	383.145	15.406	C15H29N1O6S2	1.557	0.522	8.628	0.037	0.473	0.928
8-hydroxy-(+)-δ-cadinene	220.176	9.996	C15H24O1	1.456	0.529	68.952	0.000	1.149	0.928
8-methyl thiooctyl hydroximate	221.089	2.161	C9H19N1O1S2	0.070	0.907	13.829	0.015	0.738	0.928
allantoate	176.053	1.700	C4H7N4O4	0.331	0.757	17.514	0.008	0.042	0.993
α-ethyl-L-glutamate	176.095	11.759	C7H12N1O4	3.010	0.424	32.133	0.002	0.484	0.928
benzoyl-β-D glucopyranose	284.090	14.237	C13H16O7	11.402	0.154	10.946	0.025	1.609	0.928
betalamate	211.057	5.232	C9H7N1O5	5.513	0.269	24.884	0.003	0.486	0.928
calystegine A3	160.093	4.987	C7H14N1O3	0.345	0.757	11.702	0.021	0.147	0.982

Table 2.2 continued

choline	104.106	1.096	C5H14N1O1	0.342	0.757	8.621	0.037	2.781	0.794
cocaine	304.163	4.862	C17H22N1O4	0.740	0.638	20.118	0.006	0.200	0.961
curcumin	368.118	16.744	C21H20O6	0.050	0.910	14.207	0.013	0.018	0.993
curcumin mono glucoside	530.163	13.289	C27H30O11	0.056	0.910	33.770	0.002	1.981	0.900
cyanidin 3-(p-coumaroyl)-glucoside	595.151	12.012	C30H26O13	5.663	0.269	10.779	0.025	21.243	0.080
cytidine-3-mono phosphate	323.057	1.829	C9H12N3O8P1	9.633	0.167	12.566	0.020	9.154	0.320
dalpatein	342.077	9.723	C18H14O7	1.162	0.575	65.630	0.000	0.003	0.993
dehydro spermidine	146.164	3.246	C7H20N3	44.176	0.016	32.572	0.002	55.108	0.007
delphinidin	303.051	5.213	C15H9O7	0.219	0.823	10.350	0.027	0.462	0.928
δ-cadinene	204.181	12.376	C15H24	4.373	0.327	49.035	0.001	1.338	0.928
dihydroxy feruloyl-sinapoyl spermidine	735.310	9.462	C38H45N3O12	0.000	0.998	415.800	0.000	2.407	0.837
DIMBOA	211.040	9.662	C9H8N1O5	10.047	0.163	93.649	0.000	1.186	0.928
dopaxanthin	390.114	8.887	C18H15N2O8	0.468	0.728	23.542	0.003	0.904	0.928
dTMP	322.055	1.820	C10H13N2O8P1	0.110	0.892	41.960	0.001	0.000	0.999
geranyl mono phosphate	234.110	10.448	C10H17O4P1	0.084	0.901	17.479	0.008	0.855	0.928
glucosyl-limonin	650.245	11.729	C32H41O14	1.829	0.493	32.257	0.002	0.015	0.993

Table 2.2 continued

hemigossypol-6-methyl ether	274.121	1.165	C16H18O4	3.891	0.336	62.123	0.000	4.457	0.680
indole-3-aceto hydroximoyl glutathione	480.165	6.879	C20H24N5O7S1	39.168	0.016	96.830	0.000	25.787	0.050
indole-3-acetyl iso	288.142	6.373	C16H19N2O3	1.108	0.575	45.174	0.001	0.106	0.991
indole-3-acetyl phenyl	322.131	8.528	C19H17N2O3	9.890	0.165	56.975	0.000	0.459	0.928
Indolylmethyl - desulfo glucosinolate	368.103	8.321	C16H20N2O6S1	5.821	0.269	16.817	0.008	5.674	0.500
inosine	268.085	8.749	C10H12N4O5	1.152	0.575	11.123	0.024	2.854	0.794
jasmonoyl CoA	959.234	9.811	C33H48N7O18P3 S1	0.612	0.676	87.479	0.000	1.539	0.928
L-arogenate	228.091	5.966	C10H12N1O5	4.372	0.327	54.063	0.001	0.756	0.928
leucocyanidin	306.074	6.382	C15H14O7	0.000	0.995	277.570	0.000	0.001	0.999
leuco delphinidin	322.061	1.805	C15H14O8	6.696	0.267	46.054	0.001	1.025	0.928
Mg-proto porphyrin IX 13-mono methyl ester	598.244	9.061	C35H33N4O4Mg1	1.787	0.493	11.806	0.021	1.416	0.928
medicarpin	270.092	4.942	C16H14O4	0.564	0.695	33.939	0.002	0.224	0.953
melatonin	232.116	6.891	C13H16N2O2	0.119	0.885	24.157	0.003	0.208	0.961
N-dimethyl ethanolamine phosphate	170.055	5.788	C4H11N1O4P1	2.230	0.477	72.395	0.000	1.975	0.900

Table 2.2 continued

O-aminobenz aldehyde	121.047	9.113	C7H7N1O1	2.170	0.477	83.554	0.000	0.632	0.928
OPC4 CoA	987.262	14.466	C35H52N7O18 P3S1	0.081	0.901	10.266	0.029	0.065	0.993
orcinol	124.057	1.705	C7H8O2	15.920	0.114	142.490	0.000	1.401	0.928
oxindole-3-acetyl-aspartate-N-β glucose	468.131	14.410	C20H22N2O11	4.038	0.327	133.580	0.000	0.743	0.928
oxindole-3-acetyl valine	290.121	2.688	C15H17N2O4	0.024	0.938	17.930	0.008	0.057	0.993
p- coumaroyl triacetic acid lactone	272.061	13.447	C15H11O5	5.004	0.300	21.942	0.006	12.352	0.225
palustradiene	272.256	17.914	C20H32	4.302	0.327	86.418	0.000	0.005	0.993
papaveroxine intermediate	401.154	10.355	C21H23N1O7	6.301	0.269	55.762	0.000	0.377	0.934
peonidin-3-(p coumaroyl)-rutinoside-5-glucoside	917.293	17.507	C43H49O22	5.733	0.269	14.553	0.013	6.888	0.462
phlorizin	436.138	6.205	C21H23O10	1.084	0.575	22.123	0.006	0.773	0.928
proto porphyrinogen IX	568.301	6.076	C34H38N4O4	8.305	0.190	30.408	0.002	0.776	0.928
pyrimidin-2-one ribonucleoside	228.074	5.371	C9H12N2O5	0.347	0.757	67.483	0.000	0.116	0.991
quinolinate	167.021	1.271	C7H3N1O4	4.929	0.300	48.686	0.001	0.057	0.993

Table 2.2 continued

S-[9-(methyl thio)nonyl hydroximoyl]-L-cysteine	322.136	8.764	C13H26N2O3S2	1.945	0.493	8.679	0.037	0.544	0.928
S-Inosyl-L homocysteine	386.111	8.616	C14H18N5O6S1	0.054	0.910	12.117	0.020	0.041	0.993
S-tetrahydro columbamine	341.157	5.803	C20H23N1O4	5.862	0.269	48.787	0.001	6.311	0.500
salicortin	424.125	14.135	C20H24O10	0.000	0.995	20.874	0.006	0.109	0.991
salicyl-6-hydroxy-2-cyclohexene-on-oyl	262.089	4.016	C14H14O5	15.862	0.114	11.625	0.021	11.894	0.225
salicylate-2-O-β-D-glucoside	300.091	6.021	C13H15O8	0.014	0.945	8.528	0.037	0.986	0.928
salidroside	300.113	8.041	C14H20O7	12.002	0.154	22.173	0.006	9.896	0.311
serotonin	177.100	2.548	C10H13N2O1	12.223	0.154	365.280	0.000	0.002	0.995
sesaminol	370.113	7.560	C20H18O7	5.600	0.269	9.736	0.030	7.239	0.462
tetrahydro geranyl geranyl PP	454.233	10.652	C20H37O7P2	2.302	0.477	21.344	0.006	0.542	0.928
tetrahydro palmatine	355.170	7.377	C21H25N1O4	0.441	0.739	8.922	0.036	0.574	0.928
tetrahydropteroyltri-L-glutamate	703.247	8.372	C29H33N9O12	3.268	0.393	162.450	0.000	3.519	0.726
thiamin	265.104	13.300	C12H17N4O1S1	0.020	0.939	10.010	0.029	2.830	0.794
tricoumaroyl spermidine	583.265	8.862	C34H37N3O6	0.952	0.589	13.877	0.015	4.048	0.700

Table 2.2 continued

tryptamine	161.106	5.331	C10H13N2	5.908	0.269	24.105	0.003	0.433	0.928
xanthine	152.030	1.688	C5H4N4O2	2.964	0.424	52.219	0.001	5.636	0.500

Table 2.3. Results from two-way ANOVA wheat dataset

Compound	**m/z**	**R.T.**	**Formula**	**Status F val**	**Status adj p-val**	**Cultivar F val**	**Cultivar adj p-val**	**Interaction F val**	**Interaction adj p-val**
1-18:3-2-trans-16:1 phosphatidyl glycerol	742.492	27.858	C40H70O10P1	1.773	0.505	15.246	0.001	3.509	0.348
1-O-galloyl-β-D-glucose	332.082	13.765	C13H16O10	2.627	0.385	5.122	0.043	0.920	0.714
16- methoxy tabersonine	367.205	9.737	C22H27N2O3	0.502	0.709	9.283	0.007	0.692	0.777
2-omega-hydroxy-C22:0 LPA	510.338	28.332	C25H49O8P1	19.546	0.040	13.830	0.002	1.392	0.598
24-epi cathasterone-22-O-sulfate	512.314	11.470	C28H47O6S1	8.675	0.105	18.625	0.001	9.935	0.041
3-hydroxy larreatricin	300.142	8.445	C18H20O4	0.519	0.704	5.656	0.036	2.353	0.452
3-hydroxy quinine	341.192	11.025	C20H25N2O3	5.896	0.154	6.690	0.021	0.352	0.905

Table 2.3 continued

4,8,12-trimethyl-1,3, 7,11-tri decatetraene	218.196	12.757	C16H26	13.851	0.044	2.065	0.236	0.402	0.877
4-amino butanoate	104.069	2.657	C4H9N1O2	3.240	0.303	10.344	0.005	1.002	0.689
5,6-dihydro thymine	128.049	4.257	C5H8N2O2	9.449	0.082	6.161	0.024	1.363	0.598
6-decyl ubiquinone	322.219	16.209	C19H30O4	0.004	0.997	11.501	0.004	0.391	0.880
6-methoxy mellein	208.076	8.765	C11H12O4	0.105	0.878	39.804	0.000	0.558	0.815
6-methyl sulfinylhexyl glucosinolate	465.084	4.441	C14H26N1O10S3	1.223	0.593	7.543	0.011	0.961	0.706
7,8- dihydro neopterin	255.092	4.128	C9H13N5O4	1.109	0.593	7.208	0.016	1.111	0.656
7-deoxy loganetate	198.083	7.379	C10H13O4	14.798	0.040	3.083	0.128	4.270	0.320
7-methylthio heptyl glucosinolate	463.088	4.420	C15H28N1O9S3	0.372	0.748	15.753	0.001	0.868	0.726

Table 2.3 continued

8-hydroxy abscisate	280.139	5.635	C15H19O5	0.668	0.660	10.876	0.005	0.728	0.769
8-methylthio octanal doxime	189.110	8.799	C9H19N1O1S1	12.293	0.050	9.217	0.007	1.776	0.532
9,10-epoxy-18- hydroxy stearate	314.236	17.796	C18H33O4	15.949	0.040	9.855	0.006	3.797	0.320
9-methylthio nonyl hydroximate	235.114	6.610	C10H21N1O1S2	8.408	0.105	8.547	0.007	1.353	0.598
adenosine 5'-phospho ramidate	346.079	10.714	C10H14N6O6P1	3.159	0.311	8.550	0.007	2.645	0.427
ajmaline	326.201	4.944	C20H26N2O2	0.988	0.593	17.350	0.001	0.219	0.928
alizarin	240.037	4.060	C14H8O4	1.100	0.593	4.928	0.048	0.106	0.965
α-ketol	312.222	11.899	C18H31O4	0.597	0.683	8.316	0.007	0.226	0.928
bis-β-D-glucosyl crocetin	652.268	9.748	C32H44O14	0.164	0.863	11.706	0.004	5.478	0.320
bixin aldehyde	348.203	11.608	C24H28O2	0.000	0.999	11.357	0.004	1.517	0.551
bornyl diphosphate	314.071	9.266	C10H17O7P2	15.357	0.040	2.254	0.210	3.138	0.427
coniine	128.142	3.499	C8H18N1	2.553	0.394	5.243	0.041	3.128	0.427
crepenynate	278.217	16.577	C18H29O2	0.383	0.748	4.952	0.048	1.788	0.532

Table 2.3 continued

cyanidin 3-(p-coumaroyl)-glucoside	595.151	12.012	C30H26O13	0.134	0.863	10.057	0.006	0.630	0.797
D-nopaline	306.145	4.560	C11H19N4O6	0.004	0.997	5.771	0.032	2.128	0.493
dihydro curcumin	370.133	9.022	C21H22O6	0.000	0.999	7.687	0.011	1.329	0.600
(E)-2-(methoxycarbonylmethyl) butenedioate	188.030	10.029	C7H6O6	2.298	0.429	8.068	0.011	2.521	0.427
esculin	340.072	7.838	C15H16O9	0.038	0.941	9.040	0.007	2.328	0.452
heliocide B2	424.214	10.167	C26H31O5	1.106	0.593	5.386	0.039	5.970	0.300
hemigossypol	260.107	8.859	C15H16O4	0.003	0.997	8.310	0.007	2.899	0.427
HMDBOA glucoside	387.125	6.878	C16H21N1O10	0.082	0.885	10.691	0.005	1.752	0.532
indole-3-acetyl leucine	288.152	1.666	C16H19N2O3	12.634	0.050	63.114	0.000	29.601	0.000
inosine	268.085	8.749	C10H12N4O5	1.079	0.593	5.326	0.041	1.287	0.602
iso liquiritigenin-4-glucoside	418.115	10.965	C21H22O9	0.274	0.800	4.914	0.048	1.145	0.646
laudanosine	358.198	20.898	C21H28N1O4	14.610	0.044	0.500	0.740	2.546	0.427
luteolin 7-O-β-D-glucuronide	462.072	1.660	C21H17O12	0.059	0.907	5.850	0.032	1.603	0.549

Table 2.3 continued

N-acetyl farnesyl cysteine	367.228	6.663	C20H32N1O3S1	1.003	0.593	15.031	0.001	1.188	0.640
O-sinapoyl glucarate	416.098	1.697	C17H18O12	0.000	0.999	32.549	0.000	1.177	0.640
palmatine	352.161	6.187	C21H22N1O4	0.718	0.660	8.459	0.007	2.121	0.493
pheophytin a	870.558	27.087	C55H74N4O5	0.697	0.660	8.112	0.011	0.270	0.924
phyto sphingosine	318.297	12.145	C18H40N1O3	4.072	0.230	7.374	0.016	1.255	0.615
pyridoxal	167.052	9.997	C8H9N1O3	13.398	0.044	7.730	0.011	4.285	0.320
rotenolone	410.146	6.425	C23H22O7	6.056	0.154	20.723	0.000	0.845	0.730
serotonin	177.100	2.548	C10H13N2O1	0.139	0.863	19.737	0.000	1.683	0.541
sesaminol	370.113	7.560	C20H18O7	0.000	0.999	35.386	0.000	2.752	0.427
steviol monoside	480.276	11.955	C26H40O8	13.749	0.044	3.832	0.087	2.798	0.427
stigmasterol 3-O-β-D-glucoside	574.410	11.909	C35H58O6	0.375	0.748	5.258	0.041	1.517	0.551
theophylline	180.069	4.772	C7H8N4O2	15.419	0.040	1.033	0.502	11.078	0.035
vicianose	312.103	6.271	C11H20O10	1.917	0.481	9.813	0.006	0.711	0.777
vinorine	334.167	5.658	C21H22N2O2	0.578	0.689	7.911	0.011	0.865	0.726
vitexin 2”-O-β-D-glucoside	594.149	12.039	C27H29O15	0.849	0.627	17.944	0.001	0.893	0.722
xanthine	152.030	1.688	C5H4N4O2	0.178	0.854	5.215	0.043	2.921	0.427

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CHAPTER THREE

UNTARGETED METABOLOMIC AND TRANSCRIPTOMIC ANALYSIS IN SPRING AND DURUM WHEAT REVEALS POTENTIAL MECHANISMS ASSOCIATED WITH THE EARLY STEM SOLIDNESS PHENOTYPE AND RESISTANCE TO WHEAT STEM SAWFLY

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BMC Plant Biology
Status of Manuscript:
_X_ Prepared for submission to a peer-reviewed journal
___Officially submitted to a peer-review journal
___ Accepted by a peer-reviewed journal
___ Published in a peer-reviewed journal

Abstract

Wheat stem sawfly (WSS) causes devastating yield loss in both common bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* L. var *durum*) in the North American Great Plains. The early stem solidness phenotype confers solid stems early in plant development coinciding with the flight period of WSS and provides protection to plants during the critical oviposition period. With this phenotype, pith is lost as the plant develops, which may allow for enhanced biological control of surviving larvae by braconid parasitoids *Bracon cephi* (Gahan) and *Bracon lissogaster* Muesebeck, as well as having additional potential yield benefits from utilizing reabsorbed pith components. Here, we use an untargeted transcriptomics and metabolomics approach to explore the mechanisms related to the early stem solidness phenotype in three cultivars of spring wheat and two cultivars of durum wheat in addition to three near-isogenic pairs of spring wheat and two near- isogenic pairs of durum wheat. We identified effects of growth stage and allele on expression of metabolites and transcripts associated with stem solidness, development of cell walls and programmed cell death. A caffeic acid methylesterase and pectin methylesterase were upregulated in hollow stemmed Reeder and lines with the *3BLa* allele, which likely influences lignin subunit proportion as well as the production of volatile semiochemicals that impact the behavior of adult WSS. *TaVPE3cB,* a gene associated with programmed cell death and thickening of cell walls, also had increased expression in hollow stemmed lines and is likely partially responsible for the hollow stemmed phenotype observed. Growth stage and allele also affected the expression of transcripts and metabolites involved in the phenylpropanoid pathway, carbohydrate and glycoside biosynthesis and lipid biosynthesis,

implicating the involvement of these pathways in resistance and plant response to infestation by WSS.

Introduction

Wheat stem sawfly (WSS), *Cephus cinctus* Norton, is a major pest of both common bread wheat *Triticum aestivum* L.., and durum wheat *Triticum turgidum* L. var *durum*, causing up to $350 million in yield loss annually in the Great Plains of North America (Beres et al., 2011; Weaver, 2023). First discovered in native grasses in the late 1800's, by the early twentieth century the native WSS had already adapted to use cultivated wheat as a host, and since then has chronically caused devastating yield and economic losses throughout the wheat growing regions of North America (Beres et al., 2007; Morrill et al., 1998). Adult WSS emerge from the stubble of the previous year in late spring or early summer over the period of several weeks (Wallace & McNeal, 1966). Shortly after emerging, WSS females oviposit in the wheat stem and larvae feed inside the stem until they are fully mature. Mature larvae prepare for diapause by cutting a ring around the inner stem wall at the base of the plant, plugging the hole with frass and forming a cocoon inside the space, allowing them to overwinter in the stubble below the soil surface (Beres et al., 2007). Feeding activity of the larvae damages vascular tissues and negatively affects photosynthetic ability, leading to decreased head weight and overall yield loss (Delaney et al., 2010; Macedo et al., 2005). Additionally, stem-cutting by the larvae weakens stems and causes them to lodge when exposed to wind or gravity, making harvesting difficult and leading to further yield loss (Ainslie, 1920; Beres et al., 2007; Morrill et al., 1998; Sherman et al., 2010).

Control of WSS is challenging for several reasons including the protracted flight period of adult WSS, location of the larvae within the stem and unreliable biological control options.

Since the larvae are protected inside the stem and adults emerge over a period of weeks, it is difficult to optimally time application of contact insecticide sprays and single applications are often ineffective. Systemic insecticides such as Thimet 20-G have shown some success against WSS larval infestation (Wanner & Tharp, 2015), but due to their toxicity and specific timing requirements for application, they were not widely adopted by producers and are no longer labeled for WSS control. In wheat fields, biological control exists in the form of two species of braconid wasps that are known to frequently parasitize WSS larvae. These parasitoids, *Bracon cephi* (Gahan) and *Bracon lissogaster* Muesebeck (Hymenoptera: Braconidae), reduce yield loss by increasing larval mortality (Bekkerman & Weaver, 2018; Buteler et al., 2008). Rates of parasitism from year to year are often inconsistent and can be negatively affected by the use of other WSS control methods including the planting of solid stemmed cultivars, making it an unreliable source of WSS control (Rand et al., 2012; Runyon, 2001).

Historically, the use of solid stemmed cultivars has been proven to be a partially effective management strategy for WSS. Solid stems contain pith, which is thought to slow or stop larval growth and maturation by restricting the movement of the larvae within the stem (Bathini et al., 2023; Delaney et al., 2010; Holmes & Peterson, 1961, 1962; Peirce et al., 2022). These cultivars experience lower rates of stem cutting and have the potential to reduce WSS populations over time compared to hollow stemmed cultivars (Bekkerman & Weaver, 2018; Peirce et al., 2022; Sherman et al., 2015). However, solid stems do not provide complete resistance to WSS infestation and stem cutting of 30% or more can still occur in fields planted with solid stemmed cultivars (Talbert et al., 2014). In addition, producers are sometimes reluctant to plant these cultivars as they are perceived to be lower yielding since they are thought to allocate resources to

pith production that could otherwise be utilized in grain fill (Beres et al., 2017; Weaver, 2023). These limitations of solid stemmed cultivars make the early stem solidness phenotype of particular interest, since stem solidness is expressed early in plant development but disappears as the plant matures. This provides the plant with necessary protection against infestation by WSS during early development but allows for re-allocation of resources during the critical stage of grain fill. Associated with the allele *Qss.msub-3BLc* in spring wheat, and *Qss.msub-3ALb* in durum wheat, this development coincides with the life cycle of the WSS, providing plants with protection against infestation only during the most critical stage of growth (Varella et al., 2015; Varella et al., 2019b). Despite extensive research on the solid stem phenotype, the genetic basis for the early stem solidness phenotype and the molecular mechanisms involved have not yet been explored prior to this study.

In spring wheat, the stem solidness trait is primarily associated with *Qss.msub-3BL*, a quantitative trait locus (QTL) located on the long arm of chromosome 3B which contributes 76% of the total variation for stem solidness (Cook et al., 2004). Durum wheat shares a common locus on chromosome 3B that is also associated with stem solidness, designated *SSt1* (Houshmand et al., 2007). Despite this shared locus, expression of the stem solidness phenotype differs between durum and spring wheat (Nilsen et al., 2017). In spring wheat, three alleles have been identified at this QTL: the Reeder (PI613586) (Sherman et al., 2010) allele *Qss.msub-3BLa*, which is associated with a hollow stem phenotype (Cook et al., 2019; Varella, 2016); the Choteau (PI633974) allele *Qss.msub-3BLb*, historically called the Rescue allele, which is associated with solid stems throughout development; and the Conan (PI607549) (WestBred, LLC) allele *Qss.msub-3BLc*, which is associated with stem solidness early in plant development. Near

isogenic lines with a common genetic background that differed at the 3B QTL were developed by the Spring Wheat Breeding Program at Montana State University to study the effect of this QTL on agronomic traits. The Conan allele *Qss.msub-3BLc* confers stem solidness at early stages in plant development, which disappears as the plant matures (Varella et al., 2016). This temporal expression of solid stems coincides with the time of year when WSS females are actively laying eggs and larvae are beginning to feed. Resistance due to this allele occurs both through antibiosis, by slowing larval development and causing increased larval mortality as well as through antixenosis, by causing WSS females to make fewer ovipositor insertions and lay fewer eggs (Varella et al., 2015).

In a durum wheat mapping population, a QTL was identified which was associated with early stem solidness and resistance on chromosome 3A, and near isogenic lines were developed from the resistant cultivar Pierce (PI 632366) and the susceptible landrace SD9 (PI 41353) (Varella et al., 2019b). Early stem solidness was observed in lines with the Pierce allele (*Qss.msub-3ALb*), and lines with this allele also experienced a 25% reduction in stem cutting. SD9 contributed the *Qss.msub-3ALa* allele associated with this higher incidence of stem cutting. More stem boring was observed with this allele as well, meaning that larval mortality does not happen as quickly as it does in plants with the Pierce allele (Varella et al., 2019b). In hexaploid spring wheat, a QTL on chromosome 3A was also found to be associated with larval mortality (Varella, 2016).

In recent years, “omics” technologies have been used to study gene expression and plant environmental response in hundreds of species. These technologies have broad implications for the future of genome research in many crops, including tetraploid and hexaploid wheat, but these

methods have not been used extensively to study Montana cultivars. Transcriptomics, or analysis of the gene transcripts in a system, can offer a picture of which genes may be involved in plant response to abiotic and biotic stresses, and has already been used to study several aspects of WSS resistance (Biyiklioglu et al., 2018; Y. Li et al., 2021; Zhao, 2019; Zhou et al., 2011). A comparison of hollow and solid near isogenic lines of spring wheat revealed differential expression of a gene linked to *Qss.msub.3BL* that is involved in lignin biosynthesis (Oiestad et al., 2017). Analysis of the transcriptome of spring wheat cultivars infested with WSS have also identified the involvement of the phenylpropanoid and phosphate pentose pathways in plant defense against larval feeding (Biyiklioglu et al., 2018). So far, studies have not been performed to identify sources of inherent resistance associated with the early stem solidness trait. Metabolite analysis, or metabolomics, can also be used to obtain a more complete picture of the physiological differences between resistant and susceptible plants. These small molecules are involved in every aspect of plant function and change rapidly in response to environmental changes, making them a perfect target to obtain a "snapshot" of the state of the plant at a specific point in time. WSS infestation has been found to cause changes in the metabolome of several spring wheat cultivars, particularly in the alkaloid, benzenoid and lipid chemical classes (Lavergne et al., 2020). Differences in the metabolite profiles of solid stemmed and hollow stemmed durum wheat lines have also been observed, including increased levels of osmolytes in hollow stemmed lines and variation in the concentrations of water-soluble carbohydrates in the absence of WSS infestation (Nilsen et al., 2020).

Solid stems do not completely protect against WSS infestation, and braconid parasitoids are not as effective as in hollow stemmed cultivars, which makes it difficult to incorporate solid

stemmed cultivars as part of a holistic management strategy (Buteler et al., 2015b; Rand et al., 2020; Rand et al., 2012). Early stem solidness alleles *Qss.msub-3BLc* in common wheat and *Qss.msub-3ALb* in durum wheat both lead to decreased infestation and stem cutting, with little effect on other agronomic traits, making these alleles of particular interest for the exploration of related resistance mechanisms and for the development of resistant cultivars (Sherman et al., 2015; Varella et al., 2015; Varella et al., 2019b). In this study, transcriptomics and metabolomics will be used to gain a more comprehensive understanding of mechanisms of resistance to WSS and will be used to further explore plant resistance to WSS associated with the early stem solidness phenotype.

Materials and Methods

Spring wheat cultivars Reeder and Conan and near isogenic lines (NILs) developed from a recombinant inbred line population derived from crosses between spring wheat lines Reeder and Conan as well as Reeder and Choteau were used for this study (Varella et al., 2016). Reeder is a hollow stemmed variety, containing the *Qss.msub-3BLa* allele while Conan exhibits early stem solidness associated with the *Qss.msub-3BLc* allele. The near-isogenic lines used in this study were developed by the spring wheat breeding program at Montana State University. Two NIL pairs for the 3B QTL were also used, these consisted of a resistant line with the *Qss.msub-3BLc* allele which confers early stem solidness and a susceptible line with allele *Qss.msub-3BLa* which exhibits a hollow stemmed phenotype. For the 3A QTL, Pierce, a durum wheat with early stem solidness and SD9 (PI 41353), a hollow stemmed variety were used, as well as a durum wheat NIL pair with a resistant line containing the Pierce allele, *Qss.msub-3ALb* for stem solidness and a susceptible line with the SD9 allele *Qss.msub-3ALa* (Varella et al., 2019b).

Seeds were planted in a combination of MSU mix and Sunshine mix #1 in a 50:50 by volume ratio. MSU mix contained a composite of mineral soils from the Gallatin Valley, Canadian sphagnum peat moss and washed concrete sand in a 1:1:1 by volume ratio. Sunshine mix #1 consists of a soil-less blend of Canadian Sphagnum peat moss and horticultural grade perlite. Plants were grown in a greenhouse at the Plant Growth Center at Montana State University and were fertilized and rotated weekly to ensure uniform growth. Three plants from each line were chosen at the end of stem elongation, Zadoks stage 49, and at the completion of head emergence, Zadoks stage 59. Stem tissue was collected and immediately frozen in liquid nitrogen and stored at 80°C until ready for further processing.

Plant Processing for Transcriptomics.

Frozen wheat stems were ground to a fine powder in liquid nitrogen with a mortar and pestle. Stem powder was sent to Novogene (Novogene, CA, USA) for sample preparation and analysis. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). Integrity and quantitation of RNA samples were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Library Preparation.

NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) was used according to the manufacturer's recommendations to create sequencing libraries. Sample mRNA was obtained from samples of total RNA using poly-T oligo-attached magnetic beads. The mRNA strands were fragmented using sonication with Diagenode bioruptor Pico. Reverse transcription was performed using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-) to obtain first strand cDNA. Both DNA Polymerase I and RNAse H were used for

second strand cDNA synthesis. Introduction of exonuclease/polymerases ensured that remaining overhangs were converted to blunt ends. Adenylation was performed on the 3' ends of resulting DNA fragments and NEBNext Adaptors with hairpin loop structure were ligated. Library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA) to obtain only fragments of 150-200 bp. Selected cDNA was incubated with 3µL USER Enzyme (NEB, USA) at 37°C for 15 min followed by 5 min at 95°C before performing PCR with Phusion High-Fidelity DNA polymerase, PCR primers and Index (X) Primer. The resulting PCR products were purified using AMPure XP system and Agilent Bioanalyzer 2100 system was used to assess library quality. Clusters of the samples were generated using a cBot Cluster Generation System and PE Cluster Kit cBot-HS (Illumina) according to manufacturer instructions. Finally, libraries were sequenced on an Illumina platform to generate paired-end reads.

Data Processing and Statistical Analysis of Transcriptomics Data.

Raw reads were processed through fastp to remove reads containing adapter sequences, poly-N sequences and low-quality reads. Resulting high-quality reads were used for all subsequent analyses. Reference genomes for spring wheat (IWGC RefSeq v1.0) and durum wheat (Svevo.v1) were downloaded from Ensembl release 104 (Howe et al., 2021). Reads were mapped to reference genomes using HISAT2 software. Mapped read numbers of each gene were counted using Featurecounts. Gene length and number of reads mapped to gene were used to calculate Reads Per Kilobase of exon model per Million mapped reads (RPKM). Differential expression analysis was performed using the DESeq2 R package (Love et al., 2014). P-values were adjusted for False Discovery Rate (FDR) using the approach of Benjamini and Hochberg (Benjamini & Hochberg, 2000). Differentially expressed genes were those with adjusted p-

values <0.05. Gene annotations and locations were identified using the EnsemblPlants database (http://plants.ensembl.org/Triticum_aestivum). Putative protein functions of annotated genes were characterized through the UniProt database (http://uniprot.org).

Principal component analysis (PCA) was performed in R 3.4.1 using the plotPCA function of DESeq2, ggplot2 and ggfortify packages (Love et al., 2014; Tang et al., 2016; Wickham, 2016). The dataset was subjected to variance stabilizing transformation prior to PCA.

Plant Processing for Metabolomics.

Frozen wheat stems were ground to a fine powder in liquid nitrogen with a mortar and pestle. Approximately 150 mg of stem powder was weighed and immersed in ice cold 100% methanol (MeOH). Samples were then vortexed and sonicated for 15 minutes at room temperature before centrifugation for 10 minutes at 25,000g. Proteins were separated from the supernatant by acetone precipitation with two and a half parts of acetone to one part MeOH solution at -80°C overnight, followed by centrifugation at 4°C for 10 min. The resulting supernatant fraction was dried under nitrogen gas stream and stored at -80°C. Prior to analysis by liquid chromatography-mass spectrometry (LC-MS), samples were resuspended 100 µL in 5:1 acetonitrile: water with 0.1% formic acid.

LC-MS.

Metabolite analysis was conducted at the Montana State Mass Spectrometry Facility at Montana State University in Bozeman, Montana. Analysis was performed on a Waters Synapt-XS Q-TOF interfaced with a Waters I-Class ultra-performance liquid chromatography (UPLC) (Waters Corporation, MA, USA).

Metabolites were separated by hydrophilic interaction (HILIC) chromatography on an Acquity UPLC® BEH HILIC 1.7 µm column (Waters Corporation, MA, USA) kept at 50°C with a flow rate of 400 µL min -1. The elution profile implemented started with a two-minute step of 5% solvent A (0.1% formic acid in H2O; waste) with 95% solvent B (0.1% formic acid in acetonitrile) followed by a 95% to 50% solvent B gradient over 12 min, continued 50% solvent B for 2 min, and then a return to a 50% solvent A to 95% solvent B gradient over 5 minutes. Mass detection was performed in positive mode, with a scan range from 50 – 1200 m/z with scan time of 0.2 s. Data independent analysis ("MSE"), and data-dependent analysis collision activated dissociation was performed on pooled samples; both with a ramp of 20-50 V of the collision cell. Capillary voltage was set to 2.53 V, sampling cone voltage was set to 40 V , and source offset voltage was set to 4 V. Cone gas temperature was 110°C with a flow rate of 30 L per hour -1. Desolvation temperature was 400°C with a flow rate of 500 L per hour -1. Nebulizer gas flow was 3.5 bar. Leucine enkephalin, with a reference mass of m/z 556.2771, was used as the lock mass compound.

LC-MS Data Pre-processing and Statistical Analysis.

Raw spectral data acquired in centroid mode was imported to Progenesis QI for pre-processing and compound identification (Waters Corporation, MA, USA). Each run in the experiment was compared to every other run and the run with the most similarity to all others was chosen as the reference run for automatic alignment. After review of the automatic alignment, the automatic sensitivity method was used to estimate noise levels in the data and perform peak picking across all runs. The resulting list of peaks was deconvoluted, meaning ions and adducts of the same compound were grouped together. Finally, compounds were identified

using the Metacyc database, and resulting identifications were filtered manually to remove low quality peaks and low confidence identifications (Caspi et al., 2014). Compounds were classified using the Chemical Entities of Biological Interest (ChEBI) database (Hastings et al 2015).

Statistical analysis was performed using R version 4.1.3 (R Core Team, 2022) using MetaboAnalystR (Pang et al., 2020). Log transformation and range scaling was used to meet the assumption of normality, and normalized values were used to perform a principal component analysis (PCA). A two-way ANOVA was conducted using allele and growth stage as fixed effects to identify differences in metabolic profiles of the near isogenic lines and "parental" lines containing the same alleles at Zadoks stage 49 and Zadoks stage 59. Since differences were observed between parental lines and near isogenic lines with the same allele, the data was separated into a dataset containing only near isogenic lines and used to perform PCA as well as a two-way ANOVA using allele and growth stage as fixed effects. Data from the near isogenic line pairs was also separated into three individual datasets and used to perform the same analysis.

Results

Principal Component Analysis of Transcripts in Spring Wheat.

A principal component analysis (PCA) was conducted on all 52,527 mapped genes. The first principal component explained 34% of the variance in the gene expression and showed clear segregation of the Conan and Reeder samples from the remaining samples of the near isogenic lines (Figure 3.1). Samples with the *3BLc* allele showed greater spread than the samples with the 3Ba allele.

Differential Expression of Transcripts in Spring Wheat.

Of the 1,645 genes that were differentially expressed between lines with differing alleles at the 3B QTL, 119 were aligned to chromosome 3B in the QTL region of interest containing the microsatellite marker *Xgwm340* used for development of these NILs by Varella et al. (2015) (Table 3.1). These genes had putative functions involving defense response, proteolysis, protein phosphorylation and transport and lignin biosynthesis.

TraesCS3B02G458700 was identified as a cysteine-rich peptide CRP1, a type of antimicrobial peptide (AMP) with high stability. In the Reeder samples and samples with the *3BLa* allele, the relative abundance of this transcript differed slightly between growth stages, while in the Conan samples and those with the *3BLc* allele, the variability among samples was greater at Zadoks 49 (Figure 3.2a). A 2-oxoglutarate and Fe(II)-dependent dioxygenase family gene, *TraesCS3B02G520100*, was significantly upregulated in Reeder and *3BLa* samples at Zadoks 49 and Zadoks 59 (adjusted p-value=0.003, Figure 3.2b). A possible lipoxygenase, *TraesCS3B02G602500*, was significantly upregulated in Conan and *3BLc* samples at both Zadoks 49 and Zadoks 59 (adjusted p-value=0.001, Figure 3.2c). *TraesCS3B02G603900* was identified as *TaGATA38* by Cheng et al, based on the presence of a GATA zinc finger domain (Cheng et al., 2021). Abundance of this transcript was significantly upregulated in Reeder and *3BLa* samples at both growth stages (p-value <0.001, Figure 3.2d).

TraesCS3B02G601600, a metallothionein, was identified as a candidate gene for the thickness of pith in a study of nitrogen use efficiency related traits in wheat (Zhao, 2019). Reeder samples had increased abundance of this transcript at Zadoks 49 compared to Conan and *3BLc* samples, but *3BLa* samples showed much lower transcript abundance at this stage (p-value=0.012, Figure 3.2e). Other candidate genes for stem diameter identified by Zhao (2019)

were also differentially expressed between lines with different alleles in this study, including *TraesCS3B02G608500*, an aquaporin; *TraesCS3B02G610100*, a pectin methylesterase; and *TraesCS3B02G612000*, a caffeic acid-oxy-methyltransferase. The putative aquaporin had significantly higher expression in Reeder samples at both growth stages compared to all others (adjusted p-value=0.021, Figure 3.2f). Similar expression patterns were observed for the putative pectin methylesterase (PME) and caffeic acid-oxy-methyltransferase (COMT) transcripts, both of which showed significantly higher abundance in the Reeder and *3BLa* samples (p-value=0.001, figure 3.2g-h).

3,272 genes were found to be differentially expressed between lines based on growth stage and 7 of these were located in the region of interest on chromosome 3B (Table 3.2). Putative functions for these genes included regulation of transcription and transmembrane transport, though not all were fully annotated. *TraesCS3B02G596400*, *TraesCS3B02G597000*, and *TraesCS3B02G596800* are all members of the cytochrome p450 protein family, which is the largest family of enzymes in plants. *TraesCS3B02G596400* was significantly upregulated in early growth stages across all lines (p-value= 0.0009, Figure 3.3a) which differed from the expression of the other two cytochrome p450 genes. *TraesCS3B02G597000* and *TraesCS3B02G596800* shared similar expression patterns, both were significantly upregulated in the near isogenic lines at early growth stages, and in Conan at later growth stages while no difference in expression between early and late growth stages was observed in Reeder (p-value=0.014 and 0.0267, Figure 3.3b and 3.3c respectively). *TraesCS3B02G592500*, a PIN-LIKES protein, showed differential expression at all growth stages across all lines (p-value=0.0002, Figure 3.3d). *TraesCS3B02G581900*, a putative uridylate kinase showed

increased expression in Conan and near isogenic lines with the 3Bc allele, while Reeder and lines with the 3Ba allele showed little difference (p-value=0.022, Figure 3.3e). *TraesCS3B02G583000* and *TraesCS3B02G597900*, identified as *TaVPE3cB* by Liu et al, 2023, have putative functions of protein binding and proteolysis respectively, and showed significant upregulation at later growth stages across all lines (p-value=0.035 and 0.007, Figure 3.3f and 3.3g respectively).

Neither growth stage nor allele had a significant effect on expression of *TraesCS3B02G608800*, an ortholog of *SSt1* in durum wheat (p-value= 0.66, p-value=0.09, respectively, data not shown).

Principal Component Analysis of Metabolites in Spring Wheat.

Using data from the parental lines as well as the near isogenic lines, principal components analysis (PCA) was performed on all 186 identified features, referred to here as metabolites. The first principal component (PC1), explained 28.5% of the variability in the dataset and the second principal component (PC2), explained 9.6% of the variability in the dataset. Neither PC1 nor PC2 was able to discriminate between groups of samples with the same allele or at the same growth stage (Figure 3.4).

Differential Expression of Metabolites in Spring Wheat.

Two-way ANOVA of all data from parental and near isogenic lines identified 36 metabolites which showed significant differences based on either allele or growth stage (p-value<0.05, Table 3.3). There was evidence for an interaction between allele and growth stage for 4-(4-hydroxyphenyl)-2-butanone O-[2-galloyl-6-p-coumaroylglucoside], 4-hydroxybenzeneacetonitrile, feruloyl-2-hydroxyputrescine, flavone, (-)-dioxibrassinin, kievitol, riboflavin, 3-hydroxy-4-butanolide, ethyl (S)-3-hydroxybutyrate glucoside and prunin 6"-O-

gallate (p-value<0.05). Growth stage had a significant effect on 16 metabolites, 1-O-caffeoyl-(b-D-glucose 6-O-sulfate), 3,5-digalloylepicatechin, 4-(4-hydroxyphenyl)-2-butanone O-[2-galloyl-6-p-coumaroylglucoside], caffeoylmalic acid, 1-phosphatidyl-1D-myo-inositol 3-phosphate, (+)-12a-hydroxypachyrrhizone, (-)-dioxibrassinin, sucrose, 2-O-galloylgalactaric acid and an unknown O-glycosyl compound based on growth stage (p-value<0.05). Significant differences were also observed in 26 metabolites based on allele, including 2,2,6,6-tetramethyl-4-piperidinone, feruloyl-2-hydroxyputrescine, riboflavin, kievitol, flavone, cyclocalamin, 4-(4-hydroxyphenyl)-2-butanone O-[2-galloyl-6-p-coumaroylglucoside], (-)-dioxibrassinin, santene hydrate, 3,5-digalloylepicatechin, fucose 1-phosphate, 1-phosphatidyl-1D-myo-inositol 3-phosphate, glycerol 3-phosphate, 3-hydroxy-4-butanolide, dumetorine, cinncassiol D4, an unknown O-glycosyl compound, mevalonic acid-5P, 4-hydroxybenzeneacetonitrile, an unknown benzenoid, methyl 3-(2,3-dihydroxy-3-methylbutyl)-4-hydroxybenzoate, benzoyl glucuronide, ethyl (S)-3-hydroxybutyrate glucoside, N,N'-Bis(gamma-glutamyl)cystine, semilicoisoflavone B, 8-hydroxypinoresinol, cyanidin 3-rutinoside, 4'-methyl-(-)-epigallocatechin 3'-glucuronide, prunin 6''-O-gallate and niacinamide (p-value<0.05). Of the 36 significantly different features, 30 were assigned to a class and 17 could be matched to their respective biological pathways. Most of the features were classified as carbohydrates or carbohydrate derivatives, followed by flavones, flavonoids and isoflavonoids. The majority of these metabolites were found to be directly involved in plant defense, followed by lipid/phospholipid metabolism, phenylpropanoid biosynthesis and metabolism, carbohydrate biosynthesis and metabolism and flavin/flavonoid biosynthesis (Figure 3.5).

Carbohydrate and carbohydrate derivatives including sucrose, 4-(4-hydroxyphenyl)-2-butanone O-[2-galloyl-6-p-coumaroylglucoside], an unknown O-glycosyl compound and 1-O-caffeoyl-(b-D-glucose 6-O-sulfate) generally increased at Zadoks 59, while fructose 1-phosphate appeared to decrease. Carbohydrate derivatives 3-hydroxy-4-butanolide and benzoyl glucuronide showed differing responses depending on genetic background. Flavonoids 3,5-digalloylepicatechin, cyanidin 3-rutinoside and 4'-methyl-(-)-epigallocatechin 3'-glucuronide all increased at Zadoks 59, but this increase was not significant in all genetic backgrounds. The phospholipid 1-phosphatidyl-1D-myo-inositol 3-phosphate was greater at Zadoks 59 in nearly all genetic backgrounds.

Principal Component Analysis of Transcripts in Durum Wheat.

PCA was conducted on all 46,587 mapped durum wheat genes. The first principal component explained 25% of the variance in the gene expression and showed clear segregation of the Pierce and SD9 samples from the remaining samples of the near isogenic lines (Figure 3.14). Samples with the *3ALa* allele showed greater spread than the samples with the *3ALb* allele.

Differential expression of transcripts in durum wheat.

Of the 2,128 genes that were differentially expressed between lines based on allele, 146 were matched to chromosome 3A, although just three differentially expressed genes were located within the confidence interval of the marker positions in the Svevo genome used to derive the 3A NILs (chr3A: 514130564 to chr3A: 517062504) (Avila et al., 2021; Varella et al., 2019a)(Figure 3.6, Table 3.7). These genes were *TRITD3Av1G185620*, an isoleucine-tRNA ligase, *TRITD3Av1G185730*, a senescence regulator with unknown domain and *TRITD3Av1G185910*, a MADS-box transcription factor. *TRITD3Av1G185620* and *TRITD3Av1G185910* were

significantly upregulated in hollow stemmed susceptible plants SD9 and NILs with the *3ALa* allele (p-values 0.009 and 0.00038, Figure 3.15a and 3.15b respectively).

Additionally, six differentially expressed genes on chromosome 3A outside of the NIL region matched expression profiles in comparisons of the solid stemmed cultivar CDC Fortitude and the hollow stemmed pithless1 mutant utilized in the study by Nilsen et al, 2020. Genes that were upregulated in lines with the early stem solidness allele *3ALb* included *TRITD3Av1G174790*, a gene encoding for a FAM204A protein (p-value<0.0001, Figure 3.15c), *TRITD3Bv1G198920*, a GDSL esterase/lipase (p-value=0.02, Figure 3.15d), *TRITD4Av1G252660*, a glyceraldehyde 3-phosphate phosphatase gene (p-value=0.002, Figure 15e), *TRITD6Av1G226950*, an α-1,3-mannosyl-glycoprotein 2-β-N-acetylglucosaminyltransferase gene (p-value<0.0001, Figure 3.15f) and *TRITD7Bv1G232630*, a putative cysteine protease (p-value=0.0003, Figure 3.15g). The sixth gene that shared an expression pattern with CDC Fortitude and the pithless1 mutant was *TRITD7Bv1G015280*, a gene encoding for a 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein. This gene was significantly downregulated in lines with the early stem solidness allele *3ALb* (p-value=0.035, Figure 3.15h).

Of the 3,206 genes that were differentially expressed between lines based on growth stage, 239 were matched to chromosome 3A, although just three differentially expressed genes were located within the region of interest. *TRITD3Av1G185730*, a senescence regulator protein with unknown functional domain and *TRITD3Av1G186090*, an ATP-binding cassette (ABC) transporter family protein were both significantly downregulated in lines with the *3ALb* allele (p-value=0.0219 and <0.0001 respectively, Figure 3.16a and 3.16b, Table 8) while

TRITD3Av1G186350, a malic enzyme was upregulated in these lines (p-value=<0.0001, Figure 3.16c). Outside of the 3A region, allele had a significant effect on the abundance of terpene synthase gene transcripts from chromosome 2B and 7B, *TRITD7Bv1G200640*, *TRITD2Bv1G006590*, *TRITD2Bv1G006640* and *TRITD2Bv1G051850* while growth stage had also had a significant effect on *TRITD7Bv1G200640* as well as *TRITD2Bv1G210370*.

SSt1 (*TRITD3Bv1G280530*) was identified from both the parental and near isogenic lines, but neither allele nor growth stage had a significant effect on expression (p-value=0.987 and 0.948 respectively, Figure 3.15i).

Principal Component Analysis of Metabolites in Durum Wheat.

Principal components analysis was performed on all 346 identified metabolites. The first principal component explained 43.6% of the variability in the dataset and was able to differentiate the parental lines from the near isogenic lines, while the second principal component explained 13.8% of the variability (Figure 3.17).

Differential Expression of Metabolites in Durum Wheat.

Two-way ANOVA of all data from parental and near isogenic lines of durum wheat revealed 48 metabolites that were significantly different based on stage or allele (p-value<0.05, Table 3.9). There was evidence of an interaction for fourteen metabolites including cyanidin 3-rhamnoside, 2'-hydroxynicotine, lycopersiconol, 1-(2H-1,3-benzodioxol-5-yl)-2-[2,6-dimethoxy-4-(prop-2-en-1-yl)phenoxy]propyl acetate, canarigenin 3-[glucosyl-(1->4)-6-deoxy-alloside], laudanosine, 2",4",6"-triacetylglycitin, sesaminol glucoside, PE-NMe(16:0/18:1(9Z)), an unknown annonaceous acetogenin, spinacoside D, ptelatoside B, neoreticulatacin A and cholesteryl-β-D-glucoside. All 48 metabolites were significantly different only based on specific

allele (p-value<0.05), with the exception of cyanidin 3-rhamnoside which was significantly different based on allele and growth stage (p-value<0.05). The majority of significantly different metabolites were lipids or phospholipids, followed by glycosides, terpenoids and organooxygen compounds (Figure 3.18). These metabolites were mostly involved in fatty acid and lipid biosynthesis, phosphatidylcholine biosynthesis and plant defense (Figure 3.18).

For glucosides sesaminol glucoside, cassitoroside and taxifolin 3-arabinoside, lower variability between replicates was observed at the later growth stage. DIBOA-glucoside was generally lower in the samples from Zadoks 59 and was lower in lines with the resistant Pierce allele. Lipids cholesteryl-β-D-glucoside and canarigenin 3-[glucosyl-(1->4)-6-deoxy-alloside] generally increased at later growth stages in all lines with the exception of Pierce. Lycopersiconol, the two unknown annonaceous acetogenins, and the three phospholipids showed opposite patterns of abundance in the near isogenic lines and their respective parents. 3,5-dihydroxy-6,7-didehydro-12'-apo-β-caroten-12'-al, 28-glucosyl-3b,23-dihydroxy-12,19(29)-ursadien-28-oate 3-arabinoside, spinacoside D and soyasaponin IV showed a decrease in abundance at Zadoks 59 in NILs containing the Pierce allele, while NILs containing the SD9 allele showed an increase at this growth stage. For these four metabolites, Pierce generally had the same abundance at both growth stages as NILs with the Pierce allele, but SD9 samples showed an opposite pattern to that of NILs with the SD9 allele. Furohyperforin and gibberellin A125 both had significant differences between both NIL pairs and the parental lines.

Discussion

Phenylpropanoid Pathway.

The phenylpropanoid pathway is a complex pathway responsible for the biosynthesis of many secondary metabolites including benzenoids, cinnamates, flavonoids, tannins, lignans and lignins (Dixon et al., 2002b; Strack et al., 1987a; Vogt, 2010). Phenylpropanoids and their derivatives are involved in a wide range of processes including plant-environment interactions, plant defense, and formation of structural compounds such as lignin and suberin (Dixon et al., 2002b; Fraser & Chapple, 2011). The phenylpropanoid pathway is proven to be upregulated in response to damage from insect pests such as Hessian fly and spruce bark beetle and is also induced upon fungal infection. This upregulation leads to formation of plant defense compounds as well as changes in lignin composition and cell wall fortification in leaves and stems of many species (Bi et al., 2010; Khajuria et al., 2013; Wainhouse et al., 1990). Most importantly, in recent omics studies of infested spring and winter wheat cultivars, the phenylpropanoid pathway has been implicated as part of the plant response to WSS infestation (Biyiklioglu et al., 2018; Lavergne et al., 2020).

In our complete spring wheat dataset, growth stage and allele at the 3B QTL had a significant effect on two compounds involved in phenylpropanoid biosynthesis, caffeoylmalic acid and feruloyl-2-hydroxyputrescine. Caffeoylmalic acid was found in higher abundance at the later growth stage in all NILs as well as all parental lines, while feruloyl-2-hydroxyputrescine had a variable change in abundance at the different growth stages depending on allele (Figure 8a and 8b respectively). Growth stage had a significant effect on the abundance of feruloyl-2-hydroxyputrescine while allele had a significant effect on the abundance of caffeoylmalic acid,

with no evidence for an interaction between growth stage and allele for either compound. Allele also had a significant effect on abundance of feruloyl-2-hydroxyputrescine in the NIL only dataset, but in the NIL pairs datasets only the *3BLc*/*3BLb* set showed a significant effect of allele. There was also evidence of an interaction between allele and stage in the *3BLc*/*3BLb* set for feruloyl-2-hydroxyputrescine.

Hydroxycinnamic acid amides, including feruloyl-2-hydroxyputrescine, are accumulated in response to pathogen infection, and these compounds are often associated with cell wall thickening in resistant phenotypes (Gunnaiah et al., 2012; Liu et al., 2018; Samborski & Rohringer, 1970). Increased abundance of feruloyl-2-hydroxyputrescine in wheat rachis is also involved in resistance of wheat to *F. graminearum* through the same mechanism of cell wall reinforcement (Dhokane et al., 2016). Feruloyl-2-hydroxyputrescine is induced in damaged tissues and also accumulates in systemic tissues in response to herbivory (Rivero et al., 2021). Caffeoylmalic acid also increases when plants are wounded, inducing a necrotic reaction with elicitation by salicylic acid (SA) (Housti et al., 2002). Our results show that cultivars resistant to WSS do not have higher abundance of HCAAs than susceptible cultivars, but it is possible that accumulation of these compounds could occur upon infestation with WSS, inducing cell wall changes that help defend against infestation or damage from feeding larvae.

In addition to small molecules related to phenylpropanoid metabolism, cultivar and growth stage both had significant effects on the abundance of transcripts of three genes related to lignin or pectin biosynthesis and organization in plant cell walls. *TraesCS3B02G612000*, a caffeic acid-oxy-methyltransferase involved in lignin biosynthesis was upregulated in Reeder at the early growth stage and in lines with the *3BLa* allele (Figure 2h). *TraesCS3B02G610100*, a

pectin methylesterase was upregulated in Reeder and in lines with the *3BLa* allele, with lower abundance of transcripts at the later growth stage (Figure 2g). *TraesCS3B02G596400*, a gene coding for a cytochrome p450 (CYP450) protein was significantly upregulated at the early growth stage in all lines, while other members of this protein family, *TraesCS3B02G597000* and *TraesCS3B02G596800*, were upregulated at the early growth stage in the near isogenic lines and in Conan at the late growth stage (Figure 3a-c).

Depending on the species and location in the plant, COMT genes have various effects on total lignin and can alter the structure and proportion of lignin subunits in plant tissues (Baucher et al., 2003). In wheat, COMTs have been found to be associated with the S-lignin pathway and increased lignin content in leaves and heads (Bi et al., 2010; C. Li et al., 2021). Generally, higher lignin content is associated with decreased herbivory due to physical hindrance and decreased palatability and nutrition (Santiago et al., 2013). While the cultivars in this study have not been screened for lignin content, selected hollow and solid stemmed cultivars have been used for past comparisons, and no differences were observed (McNeal et al., 1965). Despite the similarities in lignin content between solid and hollow stemmed cultivars, differential expression of these genes indicates that the Reeder allele may still cause changes in stem architecture that influence WSS behavior. In *Arabidopsis*, COMT is also weakly induced when exposed to (Z)-3-hexenol (Yoneya & Takabayashi, 2014). In planta, (Z)-3-hexenol is converted to (Z)-3-hexenyl acetate, a compound identified as a potential semiochemical in the interaction between WSS and wheat (Matsui et al., 2012; Weaver et al., 2009). Reeder has been shown to release greater amounts of (Z)-3-hexenyl acetate than Conan so increased transcript abundance of COMT may be related to this observation (Weaver et al., 2009).

In wheat, PMEs are known to play a role in resistance to *Fusarium* head blight and stem rust (*Puccinia graminis f. sp. tritici*) by influencing the pattern of pectin methylesterification in plant tissues (Lionetti et al., 2012; Wiethölter et al., 2003). PMEs are critical to cell wall accessibility of pectin and are known to affect plant susceptibility to pests and pathogens in several species. In *Arabidopsis,* a significant decrease in aphid infestation was observed in plants where PME activity was inhibited (Silva-Sanzana et al., 2019). In leaf tissue of wheat lines susceptible to stem rust, methyl esters of homogalacturonans are distributed in a blockwise fashion instead of randomly due to the action of PMEs, this structure may assist in depolymerizing and inactivating enzymes produced by invading fungi (Wiethölter et al., 2003). In several studies, high mortality of WSS larvae was observed in wheat infected by *Fusarium spp.* either through direct infection of the larvae themselves or by an unknown pathogen-induced plant response (Piesik et al., 2009; Wenda-Piesik et al., 2009). Increased abundance of the PME gene in Reeder indicates that there may be differences in the structure of stem tissues which in turn may be responsible for the increased larval mortality in Fusarium infested plants as well as the greater incidence of WSS infestation in Reeder.

Cytochrome p450s (CYPs) are involved in a diverse array of reactions including those relating to plant defense, metabolism of fatty acids and biosynthesis of antioxidants and secondary metabolites (Pandian et al., 2020). A cytochrome p450 family gene, CYP707A1 is responsible for converting abscisic acid (ABA) to caffeoylmalic acid/phaseolic acid in *Arabidopsis* roots (Geng et al., 2013). An abscisic acid alcohol was identified in our dataset, and while growth stage and allele had no significant impact on abundance, a slight decrease at later growth stages was observed for Reeder and NILs with the *3BLa* allele. CYPs in the CYP98A

subfamily are involved in monolignol biosynthesis and biosynthesis of other secondary metabolites in the phenylpropanoid pathway (Schoch et al., 2006; Sullivan & Zarnowski, 2010). The function of the cytochrome p450 genes identified here has not been determined, but transcripts for these genes were generally higher at early growth stages, while an increase in caffeoylmalic acid was observed at late growth stages. Our results indicate that ABA may be converted to caffeoylmalic acid as the plant matures, especially in Reeder and plants with the *3BLa* allele.

Other studies involving WSS resistance and plant response to WSS infestation have identified other compounds and proteins in the phenylpropanoid pathway. In an omics study of WSS infestation in Scholar and Choteau, a decrease in lignoceric acid and the protein phenylalanine ammonia-lyase was observed, indicating that plants infested with WSS larvae may experience decreased lignin formation (Biyiklioglu et al., 2018). Nilsen et al found that hollow- and solid-stemmed lines of durum wheat showed differences in the phenylpropanoids 4-hydroxycinnamate, ferulate and coumaroylquinate which were generally higher at later growth stages with variable response depending on genotype (Nilsen et al., 2020). In spring and winter wheat, Lavergne et al identified differential expression of phenylpropanoids and lignans in plants infested by WSS as well as several proteins related to the phenylpropanoid pathway, but plant response varied considerably depending on cultivar (Lavergne et al., 2020). The differing methods used across these publications limit making direct comparisons, but these results clearly show that the phenylpropanoid pathway changes in response to WSS infestation and that the response is highly variable and may depend on cultivar, growth stage, level of damage to the plant and stage of larval development.

Carbohydrates/Sugars Biosynthesis and Glycosides.

Carbohydrate metabolism in plants is complex, adaptable and highly regulated. Products of carbohydrate metabolism such as sugar and starch are important sources of energy and also serve as raw material for the biosynthesis of a wide range of organic compounds. In grain crops, carbohydrates are stored in the pith of stems during early growth and development and can be reallocated to developing heads during grain fill, increasing yield and providing the plant with protection against drought and cold stress (Blum, 1998; Saint Pierre et al., 2010). With discoveries of sugar-induced resistance genes, evidence has accumulated showing that sugars play a role as signaling molecules in response to wounding events, infection by pathogens and feeding by insect herbivores (Ahn et al., 1996; Berger et al., 1995; Schluepmann et al., 2004). Solid stem pith in spring and durum wheat is positively correlated with increased water-soluble carbohydrate (WSC) content which confers the plant with resistance to yield loss under drought conditions (Nilsen et al., 2020; Saint Pierre et al., 2010). While carbohydrate content varies considerably based on growth stage, internode and cultivar in our results and in several other studies, sugars and proteins involved in glycolysis and starch biosynthesis have also been implicated as part of the plant response to WSS infestation (Biyiklioglu et al., 2018; Lavergne et al., 2020). Additionally, it is known that WSS larvae feeding within the stem consume parenchyma and vascular tissues, and carbohydrate-rich parenchyma tissue makes up the majority of matter observed in the gut of feeding larvae (Ford et al 1979, Holmes 1949). Enzymes such as amylase, sucrase and cellulase are also present in the gut of WSS larvae, indicating that carbohydrates are hydrolyzed by the insect (Holmes 1949).

In our complete spring wheat dataset, growth stage had a significant effect on the abundance of sucrose and several saccharide-related glycosides (Figure 9a-e). Sucrose in stem

tissue was higher at later growth stages in all parental lines and most of the NIL pairs. NIL pairs which did not show an increase in sucrose at the later growth stage showed no change between the early and late growth stages. Growth stage and allele both had a significant effect on the abundance of fucose 1-phosphate, which decreased with maturity in all near isogenic lines regardless of allele (Figure 9c). In the complete durum wheat dataset, allele had a significant effect on several glycosides and oligosaccharides, while growth stage only had a significant effect on cyanidin 3-rhamnoside in the parental lines (Figure 9f-h). Significant differences in glycosides in the durum wheat dataset were mostly due to differences between the NIL pairs and the parental lines which often showed opposite responses. For example, for taxifolin 3-arabinoside increased at the later growth stage in the NILs but decreased slightly in the parental lines (Figure 9g).

While carbohydrate concentrations were variable depending on growth stage, allele and specific compound in both the spring and durum wheat datasets, they may still be involved in host plant resistance by influencing the behavior of foraging females or affecting larval performance after ingestion. Carbohydrates are an important part of insect diets regardless of species, and concentration can have variable effects depending on species. Larvae of eastern spruce budworm, *Choristoneura fumiferana*, and spruce sawfly *Gilpinia hercyniae* show preference for and perform better on a carbohydrate rich diet (Albert & Jerrett, 1981; Jensen, 1988). Positive correlations have also been observed between carbohydrate levels in maize leaves and oviposition by European corn borer, *Ostrinia nubilalis* Hübner (Derridj et al., 1986; Derridj et al., 1989). Some insect species do not perform as well when consuming a high carbohydrate diet. For example, tobacco hornworm larvae, *Manduca sexta* grew faster and had

increased body size when feeding on leaves and artificial diets with lower concentrations of glucose and fructose (Machado et al., 2015). In addition to overall carbohydrate concentration, protein:carbohydrate (P:C) ratio also has an effect on both larval performance and the behavior of adult insects. Larvae of tobacco budworm, *Heliothis virescence*, grew more slowly on artificial diets with low P:C ratio and led to decreased performance of female pupae and lower egg production in adult females (Roeder et al., 2014). Adult *Drosophila* (*D. suzukii*) also prefer to oviposit on fruits and media with a low P:C ratio (Silva-Soares et al., 2017; Young et al., 2018). There is also some evidence that P:C ratio can change the efficacy of secondary metabolites in plant resistance to insect pests (Machado et al., 2015; Perkovich & Ward, 2020; Raubenheimer, 1992). Adult WSS parasitoids *B. cephi* and *B. lissogaster* live longer and have increased egg load and egg volume when consuming carbohydrate-rich diets (Cavallini et al., 2022; Reis et al., 2019). It is possible that larvae of these parasitoid species also benefit from consuming sawfly larvae that have been feeding on plant tissue rich in carbohydrates and continue to experience benefits after becoming adults.

Other studies involving the WSS system have identified metabolites and proteins associated with glycolysis and carbohydrate biosynthesis as part of the plant response to infestation. Lavergne et al. (2020) identified a pfkB-like carbohydrate kinase and a glyceraldehyde-3-phosphate dehydrogenase, two enzymes involved in starch biosynthesis and glycolysis respectively, which were upregulated in response to WSS infestation in the mildly resistant winter wheat cultivar Hatcher. They also found that in Conan, WSS infestation caused an increase in several enzymes involved in protein biosynthesis and proteolysis along with enzymes related to carbohydrate biosynthesis, including sucrose synthase (Lavergne et al.,

2020). These results indicate that WSS infestation induces differences in total protein and carbohydrate concentration which seems to be related to plant resistance to WSS, but it is unclear whether these changes have an effect on the overall protein: carbohydrate ratio of the stem pith tissue or what effect this has on larval development. Another study of spring wheat cultivars also found that glyceraldehyde-3-phosphate dehydrogenase 1 was upregulated in the cultivars Choteau and Scholar after infestation with WSS as well as 3-phosphoglycerate a metabolite which is also involved in glycolysis (Biyiklioglu et al., 2018). In our durum wheat dataset a glyceraldehyde 3-phosphate phosphatase was upregulated in lines with the early stem solidness allele *3ALb*, although this gene is found outside the region of interest on chromosome 3A. Glyceraldehyde 3-phosphatase is not only a key component of glycolysis and glycerolipid biosynthesis, but evidence has also pointed towards its role in systemic acquired resistance (Mandal et al., 2011). The increased abundance of stem carbohydrates in solid or early solid stemmed cultivars as well as the induction of carbohydrate biosynthesis related pathways in infested plants further supports the suggestion that WSS infestation and possibly resistance are related to an overall increased energy demand by the plant, especially in resistant cultivars.

In several cereal crops, differences in WSC are also associated with changes in stem solidness, plant developmental stage and specific internode which are all factors that influence the oviposition behavior of WSS females (Ford et al., 1979; Nagata et al., 2002; Saint Pierre et al., 2010; Teulat et al., 2001; Thevenot et al., 2005). WSC are found in pith tissue with content of these carbohydrates higher in lower internodes compared to the peduncle or upper internode (Li et al., 2015; Robinson et al., 2007). Since these carbohydrates are found in the pith, concentrations are often higher in solid stemmed cultivars, a phenomenon also observed in the

spring wheat cultivars used in this study (Ford et al., 1979; Nilsen et al., 2020). In both hollow and solid stemmed wheat cultivars, stem carbohydrates rapidly increase until several days after anthesis, when concentration declines (Ford et al., 1979). Female WSS choose to oviposit in the lowest internodes available that are actively elongating, where carbohydrate content is likely higher. They also commonly lay eggs in solid stem cultivars where larvae do not perform as well, but which do have increased WSC content compared to hollow stemmed cultivars, which suggests that WSC and other carbohydrates in the stem may be related to host plant selection and host plant resistance (Holmes & Peterson, 1960).

Lipids/Phospholipids.

Plant lipids are structurally diverse and therefore play many roles in the plant system in addition to energy and carbon storage. In plants, lipids act as structural components, signaling molecules, and precursors to compounds such as jasmonic acid that are involved in plant defense (Lavell & Benning, 2019; Reymond & Farmer, 1998; Suh et al., 2022). Phospholipids, galactolipids and sphingolipids are components of cell membranes that can sometimes act as signaling molecules under stress conditions (Laxalt & Munnik, 2002; Munnik et al., 1998). Lipids are also distributed in the thylakoid membrane and play a functional role in the assembly of photosynthetic complexes PSI and PSII (Yoshihara & Kobayashi, 2022).

Not only are lipids essential to plant growth and development, their involvement in host plant resistance and plant-insect interactions has also been well studied. Lipids are required by both plants and many insects that feed on them, and lipids and genes related to lipid biosynthesis and degradation change in response to insect infestation in many plants, although these changes are often highly variable depending on plant and insect species as well as lipid class (Khajuria et

al., 2013; Kosma et al., 2010; Marti et al., 2013; Zhou et al., 2011; Zhu et al., 2012b). Lipids are necessary for the normal growth and development of many insect species and are often acquired through feeding on plant material (Turunen, 1979). There are many insect species that store lipids prior to diapause, and these lipid stores are used throughout the overwintering period and are associated with increased larval survival (Lehmann et al., 2020; Sinclair & Marshall, 2018). Conversely, high concentrations of lipids in larval diets can also slow larval growth and even cause mortality in some species (Li et al., 2014; Liao et al., 2021).

In our complete spring wheat dataset, both allele and growth stage had a significant effect on the abundance of 1-phosphatidyl-1D-myo-inositol 3-phosphate, a compound involved in sphingolipid biosynthesis which increased at the later growth stage in all lines, regardless of allele (Figure 10a). Additionally, glycerol-3-phosphate, a molecule involved in glycolysis and the first step of glycerolipid biosynthesis, decreased significantly at the later growth stage (Figure 10b). In the complete durum dataset, allele had a significant effect on the phospholipids PE-NMe(16:0/18:1(9Z)) and PE-NMe(16:0/16:0) (Figure 10c-d). In both parental lines and NILs containing the Pierce allele, abundance of PE-NMe(16:0/18:1(9Z)) and PE-NMe(16:0/16:0) decreased at the later growth stage, while in NILs containing the SD9 allele, these phospholipids increased with maturity. *TRITD4Av1G252660*, a glyceraldehyde 3-phosphate phosphatase gene was also significantly upregulated in lines with the early stem solidness allele *3ALb* (Figure 15e).

Other studies investigating stem solidness, the WSS tritrophic system and specifically plant response to WSS infestation have also identified differences in lipids, phospholipids or lipid-like compounds between cultivars. In comparisons of a *pithless* mutant and solid stemmed cultivar CDC Fortitude higher amounts of several lipids were identified in CDC Fortitude

(Nilsen et al., 2020). Glycerol-3-phosphate also decreased with maturity regardless of cultivar (Nilsen et al., 2020). A study using infested spring and winter wheat showed that in the resistant cultivar Conan and mildly-resistant Hatcher there was an increase in some lipids and lipid-like molecules after WSS infestation, while the opposite effect was observed for other lipids (Lavergne et al., 2020). This variability in lipids across cultivars and in response to WSS infestation is not unique among plant-insect systems. In wheat isogenic lines resistant to the stem gall-forming Hessian fly, the total concentration of many lipids decreased during incompatible interactions while susceptible plants showed a slower and less marked decrease (Zhu et al., 2012b). The opposite response is observed in maize, where phospholipids and several free fatty acids increased in response to Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisduval) (Marti et al., 2013). Membrane lipids were reduced in resistant wheat infested with Hessian fly while free fatty acids and several intermediates in the phospholipid pathway increased, including glycerol-3-phosphate, an inducer of systemic acquired resistance (Chanda et al., 2011; Khajuria et al., 2013). Genes involved in lipid metabolism were upregulated while others were downregulated in rice infested with rice stem borer, *Chilo suppressalis* (Walker) a few days after introduction of the pest (Zhou et al., 2011). The results from these studies illustrate the variable nature of lipids related to insect resistant phenotypes and the need for further study of specific lipid response to WSS infestation.

Other Spring Wheat Genes.

Abundance of *TraesCS3B02G603900* (*TaGATA38*), was significantly upregulated in Reeder and *3BLa* lines at both growth stages, while Conan and lines with the *3BLc* allele showed no difference (Figure 2d). In *Arabidopsis*, orthologs of *TaGATA38* are associated with seed

germination, flowering and response to cold stress (Richter et al., 2013). An ortholog of *TaGATA38* in Brassica napus also had lower expression under drought and cold stress (Zhu et al., 2020). GATA genes in *Brachypodium* were also found to be sensitive to treatment with methyl jasmonate and salicylic acid and overexpression of *BdGATA13* in *Arabidopsis* increased chlorophyll content in leaves (Guo et al., 2021; Peng et al., 2021). In wheat, more than 75 GATA genes have been identified and classified into at least four subfamilies (Cheng et al., 2021; Feng et al., 2022). GATA genes in wheat show high diversity in expression under abiotic stresses depending on plant tissue, but *TaGATA38* has been found to have reduced expression in response to cold stress and phosphorus starvation and increased expression in response to heat and drought stress (Feng et al., 2022). While it has been proposed that GATA genes are responsible for regulating downstream genes involved in cold and drought stress, the exact mechanisms for the observed resistance to abiotic stresses have not yet been elucidated so it is unclear whether physiological changes that occur with overexpression of *TaGATA38* are related to increased susceptibility to WSS in Reeder and lines with the *3BLa* allele.

In the spring wheat dataset, *TraesCS3B02G597900* (*TaVPE3cB*) was significantly upregulated at Zadoks 59 across all lines. Increased expression was also observed in hollow stemmed lines with the *3BLa* allele (Figure 3g). Recently, *TraesCS3B02G597900* was identified as a candidate gene for pith thickness at plant maturity by Liu et al and named *TaVPE3cB* (Liu et al., 2023). Similar to our results, Liu et al found that this gene showed significant expression differences between the early stem elongation stage (Zadoks 32) and mid-anthesis (Zadoks 65) with the highest expression in stems of a low pith thickness/hollow cultivar (Liu et al., 2023). *TaVPE3cB* is a member of the VPE3 gene family and codes for a vacuolar processing enzyme

that is highly expressed in stem tissues at the stem elongation stage (Liu et al., 2023). In monocots, VPEs are often classified into two subgroups, one group contains VPE genes which are expressed in seeds and the other contains genes which are specific to leaves and stems. While there are exceptions, VPEs found in leaves and stems (α-VPE and γ-VPE) are generally associated with lysis while seed type VPEs (β-VPEs) are associated with storing various types of proteins (Vorster et al., 2019). In *Arabidopsis*, γ-VPE was found to be expressed in the xylem and phloem of developing stems and is involved in programmed cell death (PCD) and thickening of secondary cell walls through activation of CEP1 proteases (Cheng et al., 2019). It is therefore possible that the increased expression of *TaVPE3cB* in hollow stemmed lines at the later growth stage may be responsible for PCD and the hollow stemmed phenotype observed.

Other Durum Wheat Genes.

TdDof (*TRITD3Bv1G280530*), coding for a Dof zinc finger protein, was previously found to be responsible for the majority of variation in stem solidness in both durum and spring wheat mapping populations and is a strong candidate gene for *SSt1*. Increased copy number variation (CNV) of this gene observed was observed in solid stemmed lines, with the exception of a single solid stemmed Australian cultivar ‘Janz’ which had just one copy (Nilsen et al., 2017; Nilsen et al., 2020). Their results suggest regulation of genes involved in programmed cell death by *TdDof* during early stem elongation in hollow stemmed cultivars leading to loss of pith parenchyma tissue (Nilsen et al., 2020). While our study focused on the 3A QTL in durum wheat associated with early stem solidness and not the 3B QTL where *TdDof* is located, an increase in transcripts of *TdDof* was observed in the parental lines although this difference was not statistically significant. Between Zadoks stage 49 and 77, the early stem solidness observed in Pierce during

early stem elongation (Zadoks 34-37) disappears, leaving it with stem solidness similar to susceptible cultivars of both durum and spring wheat (Varella et al., 2019b). In our study, we observed that the transcript abundance of *TdDof* decreased with plant maturity in both Pierce and SD9. Expression was slightly higher in solid stemmed Pierce samples although neither allele nor growth stage had a significant effect on the gene expression of *TdDof1* (Figure 3.15i).

Other research identified *TraesCS3B02G608800*, an ortholog of *TRITD3Bv1G280530* in spring wheat, as *TaDof3.2-3B*. They found that expression of this gene was generally low in several cultivars of spring wheat and in most plant tissues did not change much through plant development or in response to biotic and abiotic stresses including infection with *F. graminearum* or drought, heat and cold stress (Fang et al., 2020). It was found that *TraesCS3B02G608800* was highly expressed at early stem elongation, especially in the solid stemmed cultivar 'Westonia', an expression pattern that was also observed in durum wheat (Liu et al., 2023; Nilsen et al., 2020). In our results, expression of the ortholog of *TRITD3Bv1G280530* in spring wheat, *TraesCS3B02G608800*, also decreased from Zadoks 49 to 59 in hollow stemmed Reeder and NILs with the *3BLa* allele (Data not shown). However, Conan displayed a slight increase in expression from Zadoks 49 to 59 and NILs with the *3BLc* allele experienced a slight decrease in transcript abundance at the later growth stage. Conan also had lower expression of *TraesCS3B02G608800* than Reeder, indicating that this gene may not be solely responsible for the early stem solidness phenotype seen in Conan. While stem solidness continues to decrease in Conan from Zadoks 49 to head emergence (Zadoks 59), the loss of stem solidness from early stem elongation (Zadoks 35) to head emergence is much more drastic, a trend also observed in the durum wheat cultivar Pierce which also exhibits early stem solidness

(Varella, 2016; Varella et al., 2019a). This means that it is possible that Conan and Pierce may have higher expression of *TaDof/TdDof* at the start of stem elongation that we were unable to capture as it occurred prior to our collection of stem samples at Zadoks 49.

In the durum wheat dataset *TRITD3Av1G185620*, an isoleucine-tRNA ligase and *TRITD3Av1G185910*, a MADS-box transcription factor, were significantly upregulated in the hollow stemmed cultivar SD9 as well as NILs with the *3ALa* allele (Figure 15a-b). These transcripts were also found in higher abundance in hollow stemmed lines and *pithless1* mutants in a previous whole genome sequencing analysis aimed at identifying candidate genes for *SSt1*, although the differences were not found to be significant (Nilsen et al., 2020). This indicates that in durum wheat, *TRITD3Av1G185620* and *TRITD3Av1G185910* may be associated with the hollow stemmed phenotype, at least in early development. Although MADS-box transcription factors have not been well characterized in durum wheat, genome wide identification and characterization of this gene family has been performed in bread wheat, most recently with the publicly available reference genome IWGSC Ref. Seq v1.1 (Ma et al., 2017; Raza et al., 2022). The durum wheat gene *TRITD3Av1G185910* shares 100% sequence homology with hexaploid wheat gene *TraesCS3A02G284400*, a MIKC-type MADS-box gene that is typically highly expressed in the apical meristem and is known to be involved in regulation of the flowering stage (Yang et al., 2021). A strong positive correlation between this gene and abundance of the phenolic compound quercetin-3-O-rutinoside (rutin) has also been observed in hexaploid wheat, pointing at an association between this gene and high antioxidant activity (Ma et al., 2022). While rutin was not found in the durum wheat dataset, analysis of the complete spring wheat dataset revealed that growth stage had a significant effect on the related rutinoside cyanidin-3-

rutinoside. Phenolic compounds and other flavonoids are reported to be involved with feeding and oviposition behaviors in many plant-insect systems (Simmonds, 2001), and rutin in particular is associated with increased feeding and oviposition in many insect species including *Heliothis zea* and *Helicoverpa armigera*, although behavioral response can vary depending on the growth stage of the insect and flavonoid concentration (Blaney & Simmonds, 1983; Ohsugi et al., 2014). Rutin has also been shown to inhibit the growth and survivorship of European gypsy moth *Lymantria dispar* L. and European corn borer *Ostrinia nubilalis* Hübner (Abou-Zaid et al., 1993; Beninger & Abou-Zaid, 1997). Further characterization of the effects of these genes in hollow stemmed durum and spring wheat cultivars may determine how they are associated with susceptible phenotypes.

Conclusion

While the solid stem phenotype is widely used to protect against crop loss due to wheat stem sawfly, expression of solid stems can be variable depending on environmental conditions and braconid parasitoid populations are also negatively affected by the continued use of solid stemmed cultivars. These drawbacks mean that despite the success of solid stemmed cultivars as a control measure against WSS, extensive yield loss and stem cutting can still occur, and it is necessary to explore additional sources of resistance such as the early stem solidness phenotype. Early stem solidness is a phenotype that offers protection to the plant through the time of flight and oviposition period of WSS, while also allowing for better support of populations of braconid parasitoids since pith is lost as the plant develops. Assessing transcriptomic and metabolomic responses in cultivars with early stem solidness such as the spring wheat cultivar Conan and the durum wheat cultivar Pierce will help elucidate the mechanisms related to the early stem

solidness phenotype and lead toward development of cultivars that can be used to strengthen the tritrophic interaction between wheat, WSS and their parasitoids and prevent yield loss.

Here we identified effects of growth stage and allele on expression of metabolites and transcripts associated with the phenylpropanoid pathway. In the complete spring wheat dataset *TraesCS3B02G612000*, a caffeic acid methylesterase and *TraesCS3B02G610100*, a pectin methylesterase, were both upregulated in Reeder and lines with the *3BLa* allele, suggesting that these genes may be affecting total lignin content, lignin subunit proportion, cell wall accessibility of pectin or production of volatile semiochemicals such as (Z)-3-hexenyl acetate that affect WSS behavior. Several other studies involving stem solidness or the WSS tritrophic system have implicated metabolites, proteins and genes associated with the phenylpropanoid pathway, however expression is affected by genotype and is highly variable even in the absence of WSS infestation. While the role of the phenylpropanoid pathway in WSS resistance has yet to be fully characterized, the possible structural differences in stem tissue on the molecular level may be responsible for the resistance of some cultivars to WSS infestation and should be explored further.

Differences in carbohydrate abundance were observed in both the spring and durum wheat datasets, but the patterns of abundance varied depending on carbohydrate type, growth stage and allele. WSS infestation has been shown to have an effect on carbohydrate abundance, where responses differ depending on cultivar and specific carbohydrate type (Biyiklioglu et al., 2018; Lavergne et al., 2020). Water soluble carbohydrates (WSC) were found in higher abundance in solid stemmed cultivars, matching previous associations of WSC content with stem solidness in uninfested plants (Nilsen et al., 2020). WSS larvae feed on parenchyma tissue which

is where the majority of carbohydrates are located inside the stem, and despite the variability in carbohydrate concentration between cultivars, the ratio of proteins to carbohydrates in consumed tissue can affect larval development in a variety of ways. Effects of carbohydrates in the diets of WSS larvae can also carry over to other trophic levels and influence the fitness of future parasitoid generations. Unfortunately, protein: carbohydrate ratios in insect diets can have either positive or negative effects depending on species and specific concentrations of proteins and carbohydrates so additional investigation is needed to determine whether there are differences in the protein: carbohydrate ratio of stem tissue in resistant lines and the effects these ratios might have on WSS growth and development.

Growth stage and allele both had significant effects on lipids identified in the spring and durum wheat datasets. In spring wheat, an intermediate of the sphingolipid biosynthesis pathway, 1-phosphatidyl-1D-myo-inositol 3-phosphate, increased at the later growth stage while an intermediate of glycerolipid biosynthesis, glycerol-3-phosphate decreased. The durum wheat dataset showed several phospholipids which decreased with plant maturity in the resistant line. Induced changes to the lipidome and the subsequent effects on insect pests are highly variable in other plant-insect interactions but can affect larval development. The effect of the lipidome on WSS larvae has not been characterized, but it is possible that lipid profiles are associated with resistance or susceptibility to WSS.

From our transcriptome analysis, genes associated with stem solidness, programmed cell death and cell wall development were also identified, including *TaVPE3cB*, *TdDof* and *TaDof*. Increased expression of *TaVPE3cB*, a gene associated with increased programmed cell death and cell wall thickening was observed in Reeder and lines with the *3BLa* allele indicating that this

gene is likely responsible for the hollow stemmed phenotype in Reeder. *TdDof*, previously identified as a candidate gene for *SSt1* in durum wheat, was not differentially expressed between hollow stemmed lines and lines with the early stem solidness allele in our study. *TaDof*, ortholog of *TdDof* in spring wheat, decreased at the later growth stage in Reeder and lines with the *3BLa* allele, while Conan experienced a slight decrease and overall lower expression than the hollow stemmed lines. Most solid stemmed cultivars have increased copy number variation of *TdDof*, however the expression observed in Pierce and Conan seems to suggest that *TdDof/TaDof* is not the only gene responsible for the early stem solidness trait.

Although there is high variability in the metabolome, proteome and transcriptome of solid stemmed cultivars, hollow stemmed cultivars and cultivars that exhibit early stem solidness, there is a consensus that the phenylpropanoid pathway, carbohydrate biosynthesis and programmed cell death are likely involved in resistance and plant response to WSS infestation. Differences in metabolites and genes involved in lignin biosynthesis and subunit composition as well as variability in carbohydrates and lipids demonstrate the need for further characterization of molecular differences in the stem architecture of resistant cultivars as well as the effect of tissue composition on the behavior and development of WSS. Additionally, further exploration of the 3B QTL and previously identified candidate genes for *SSt1* will help identify the mechanism responsible for regulating early stem solidness.

Acknowledgements

Funding for the Montana State Mass Spectrometry Facility used in this publication was made possible in part by the MJ Murdock Charitable Trust, the National Institute of General Medical Sciences of the National Institutes of Health under Award Numbers P20GM103474 and

S10OD28650, the MSU Office of Research and Economic Development, and the Montana Wheat and Barley Committee. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Figure 3.1 Principal component analysis of spring wheat transcripts

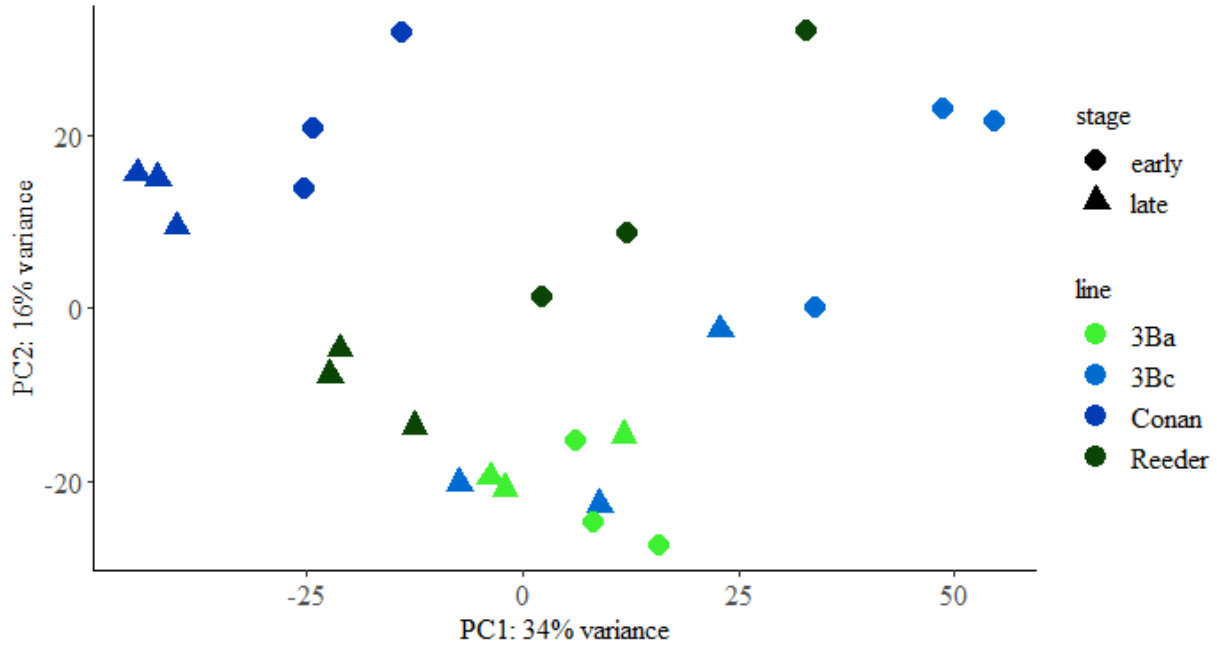


Figure 3.2 Differential expression of spring wheat transcripts with significant allelic differences

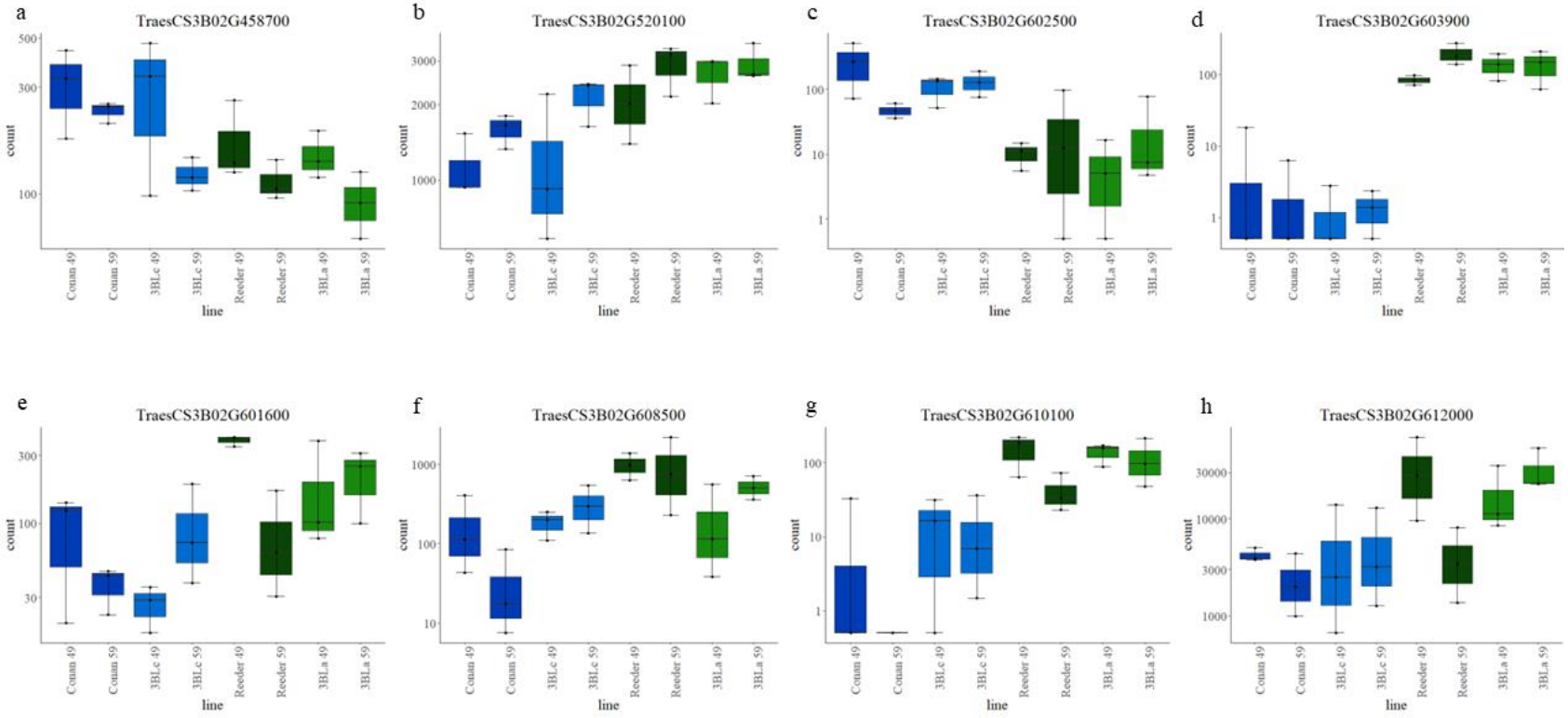


Figure 3.3 Differential expression of spring wheat transcripts, with significant growth stage differences

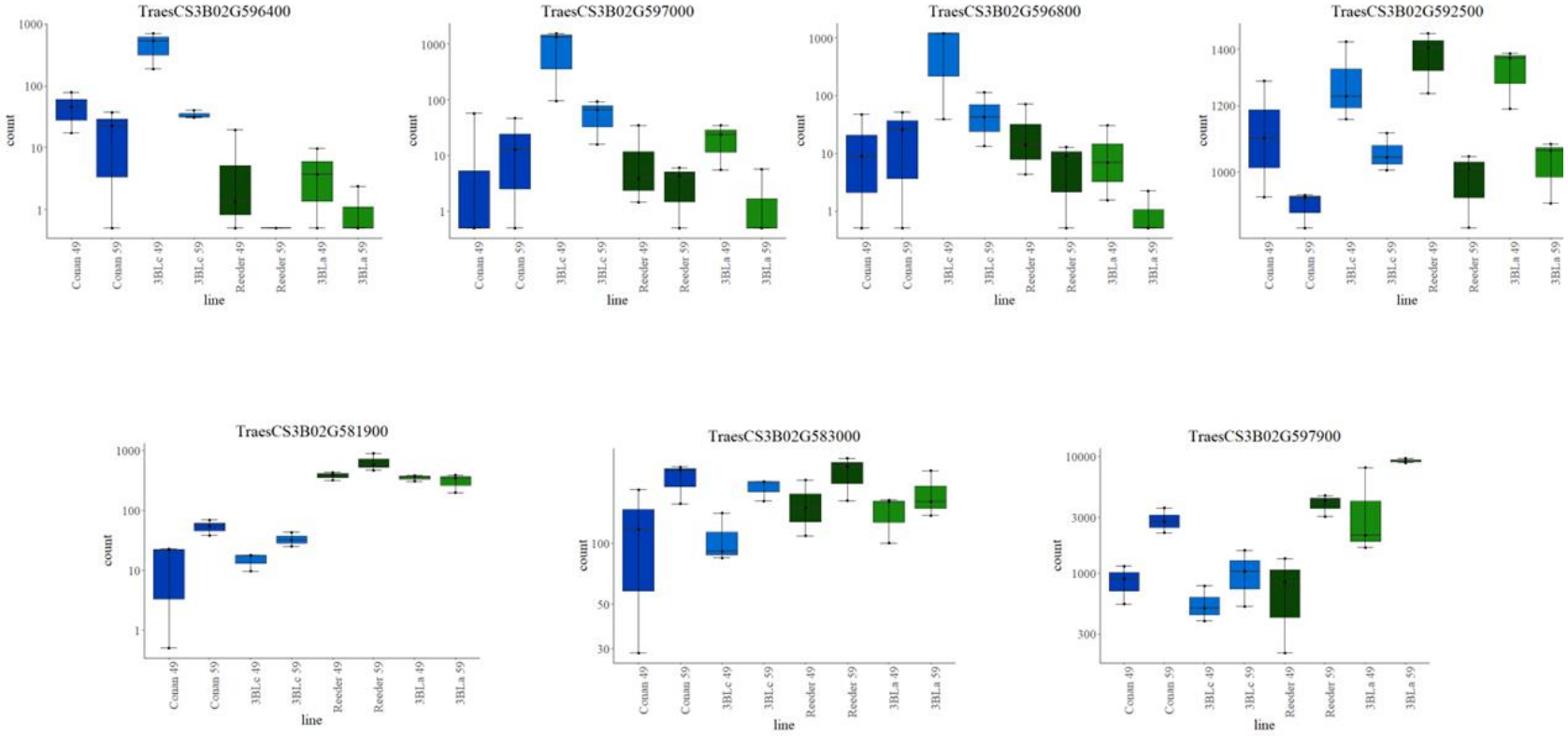


Figure 3.4 Principal component analysis of spring wheat metabolites

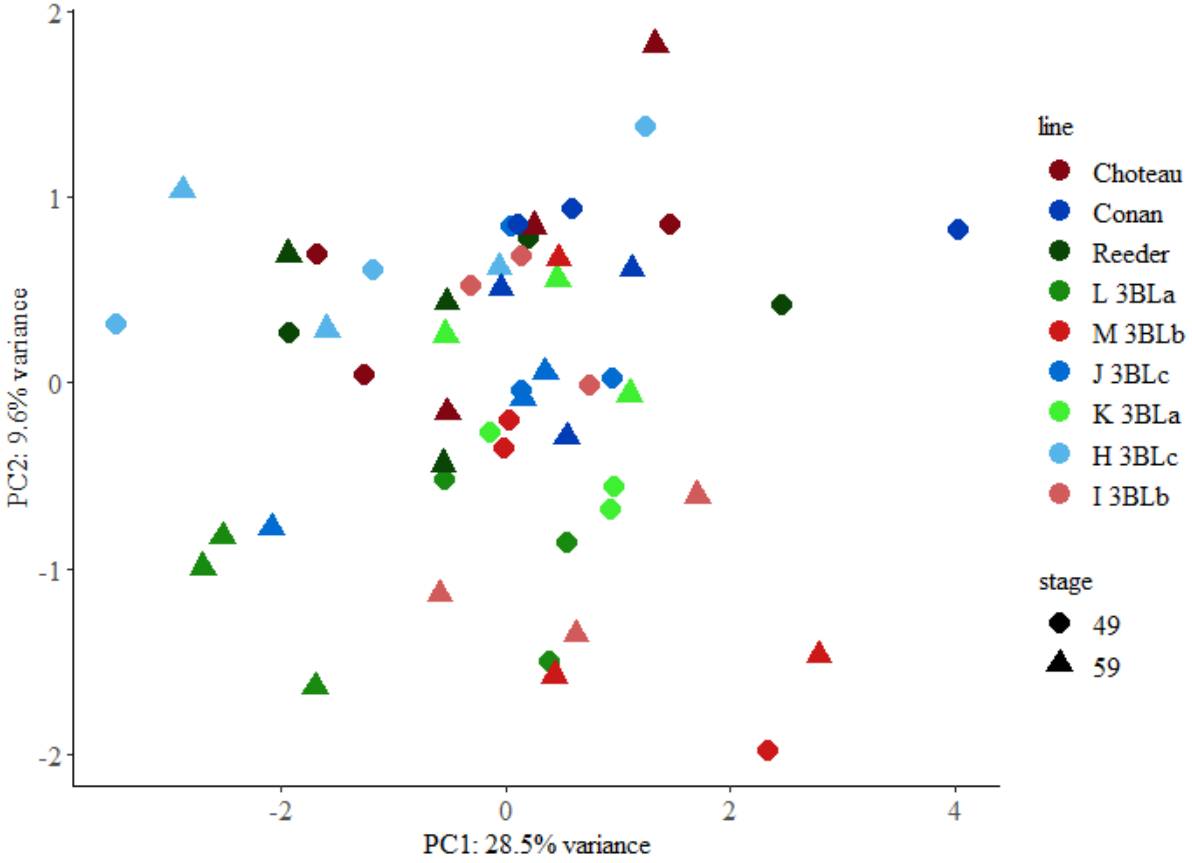
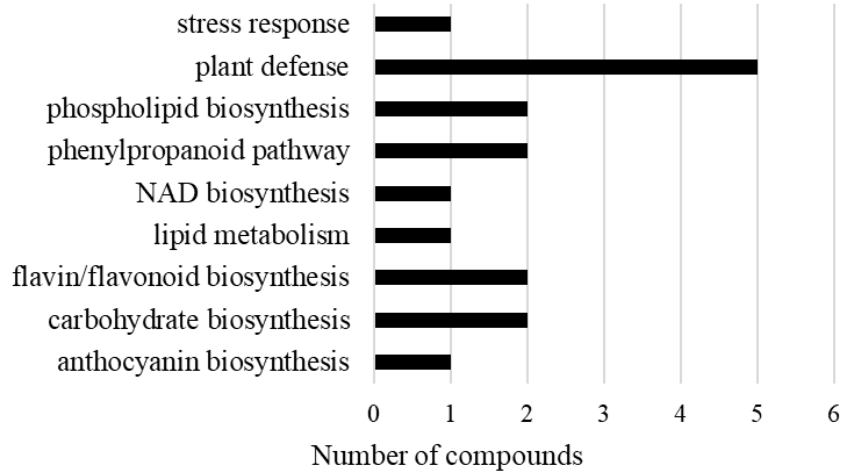


Figure 3.5 Pathway associations and classifications of significant compounds in spring wheat



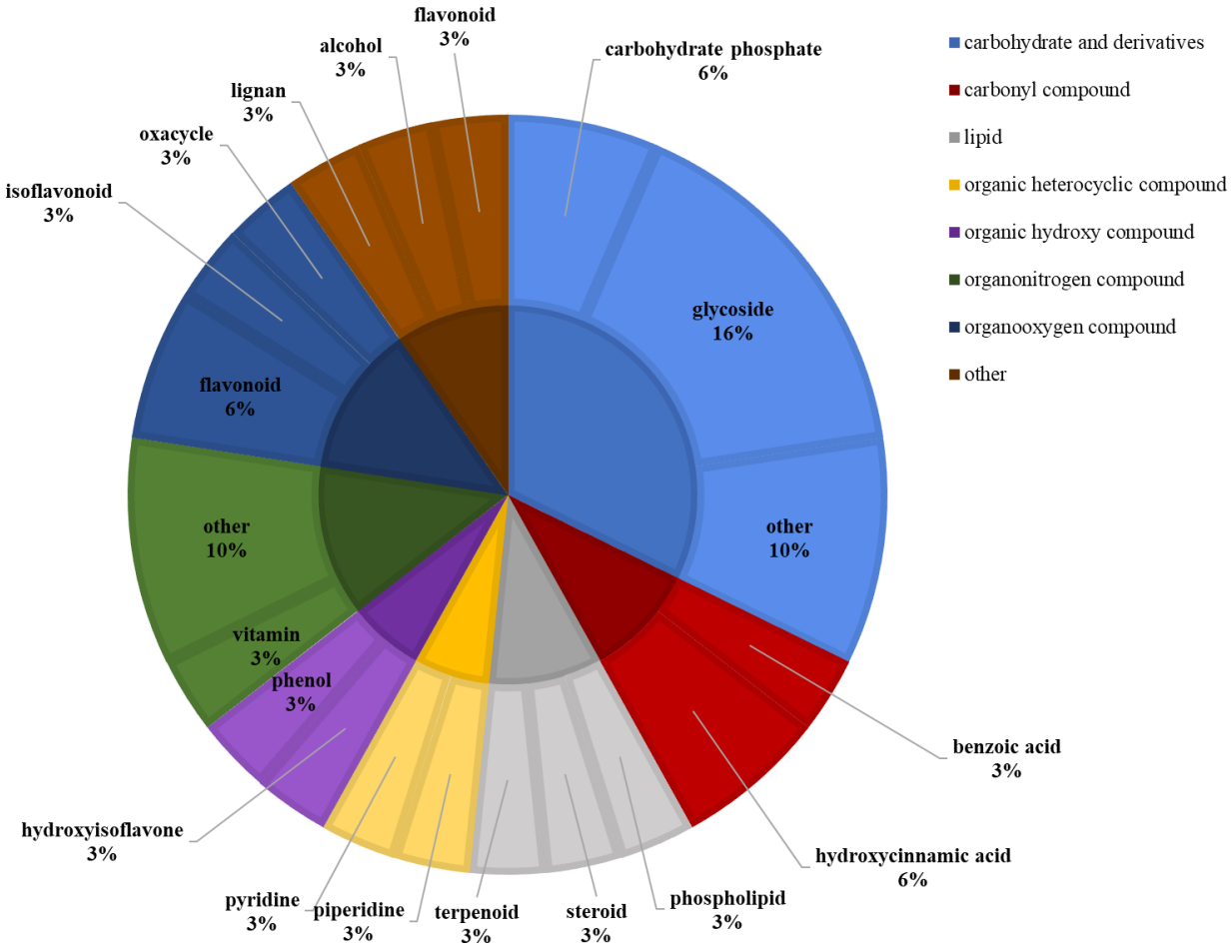


Figure 3.6 Principal component analysis of metabolites in spring wheat near isogenic lines

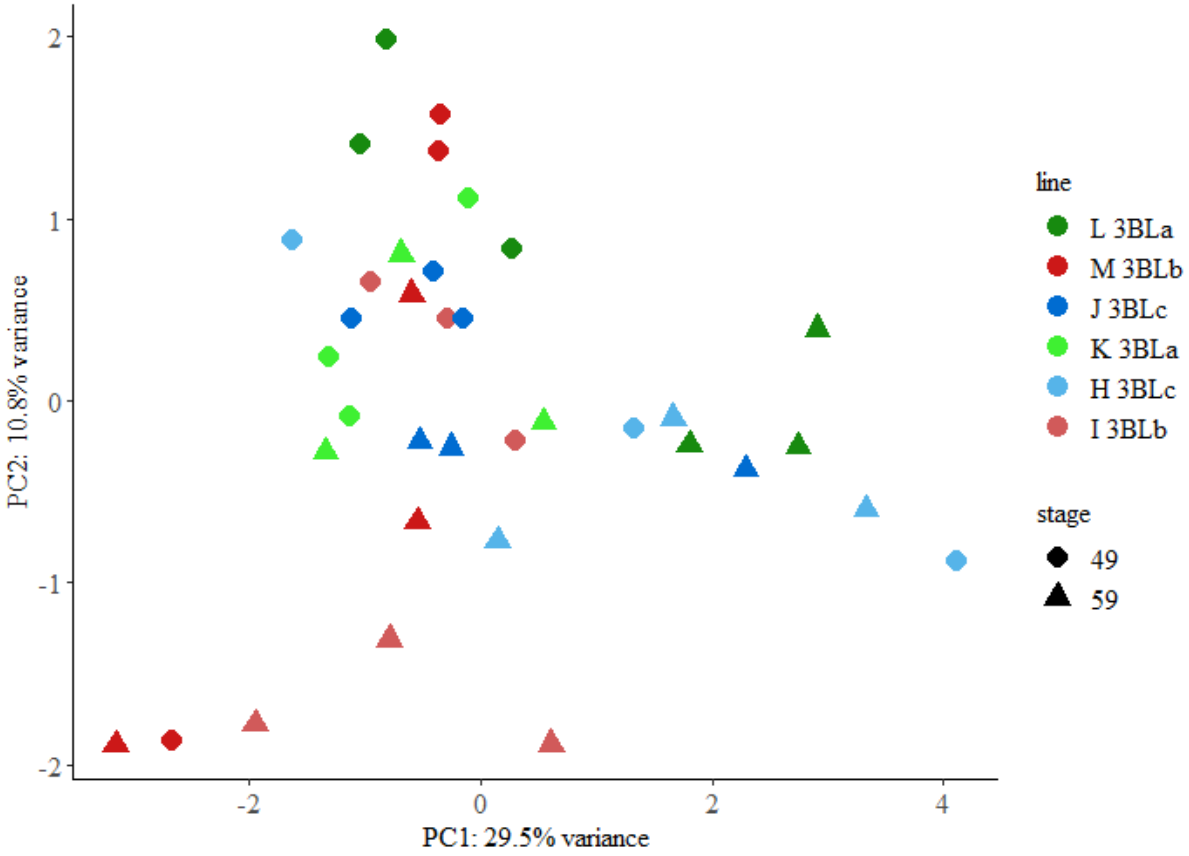
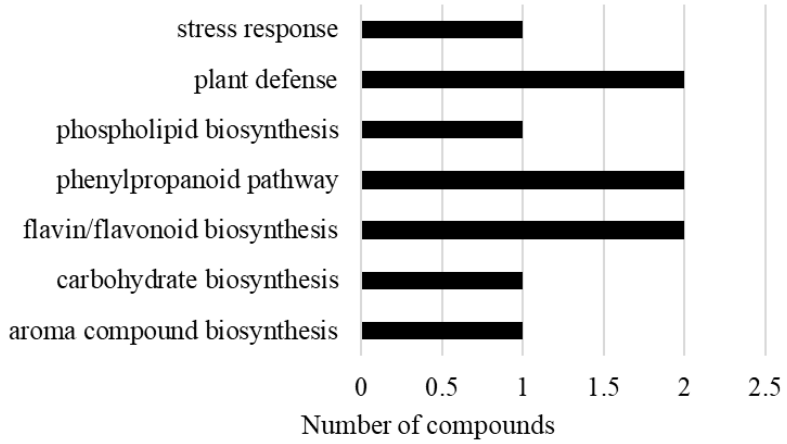


Figure 3.7 Pathway associations and classifications of significant compounds in spring wheat NILs



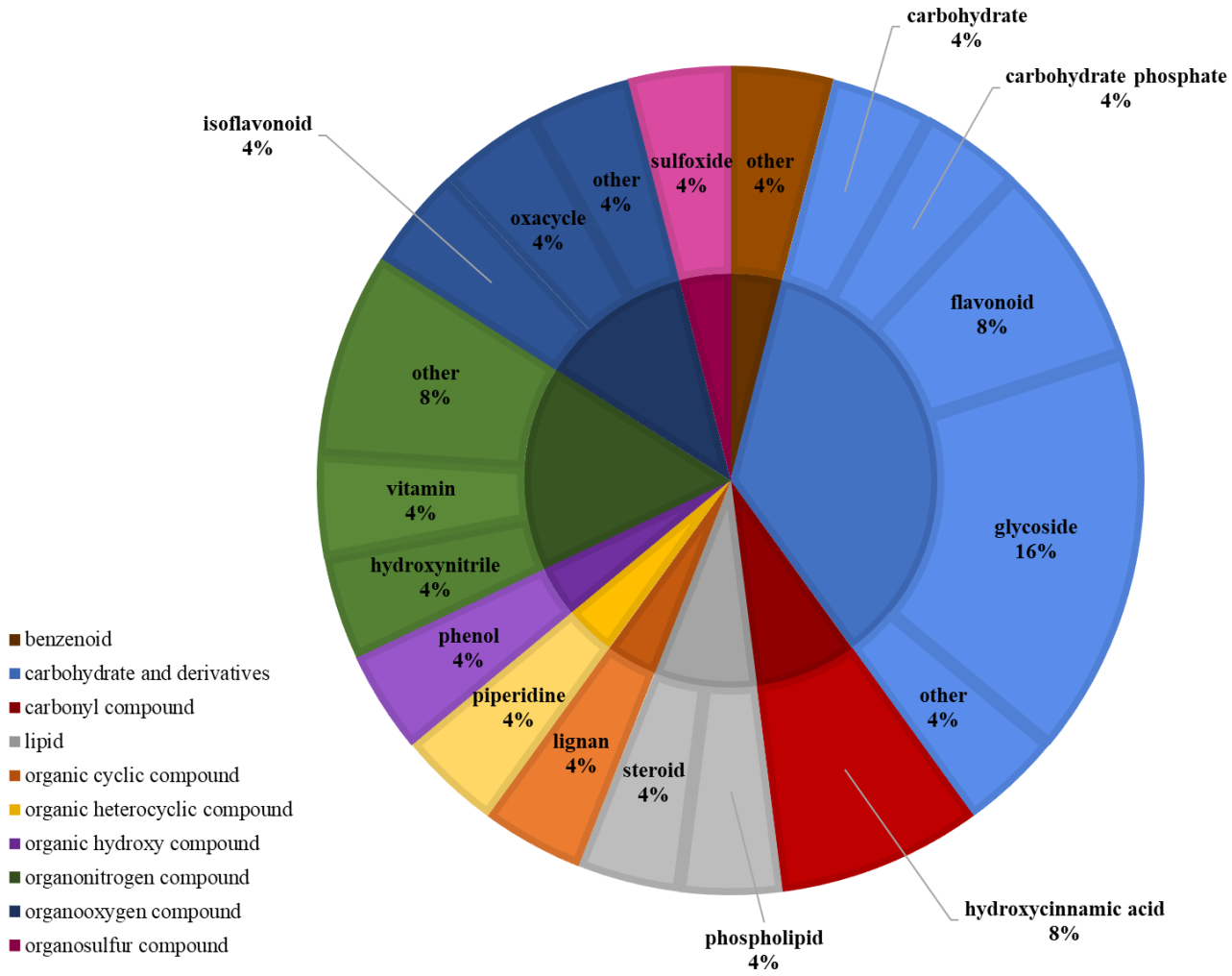


Figure 3.8 Relative abundance of phenylpropanoid pathway related metabolites in spring wheat

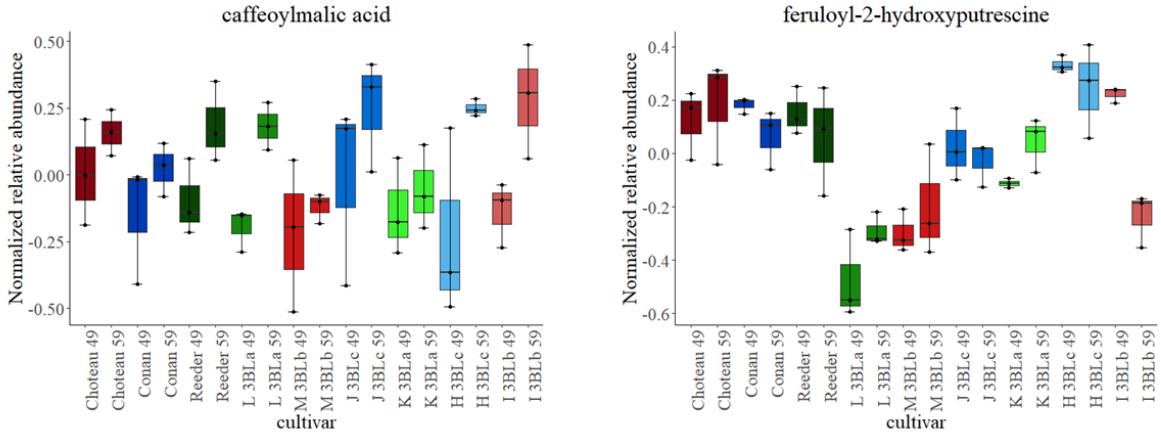


Figure 3.9 Relative abundance of carbohydrates and related glycosides in spring and durum wheat

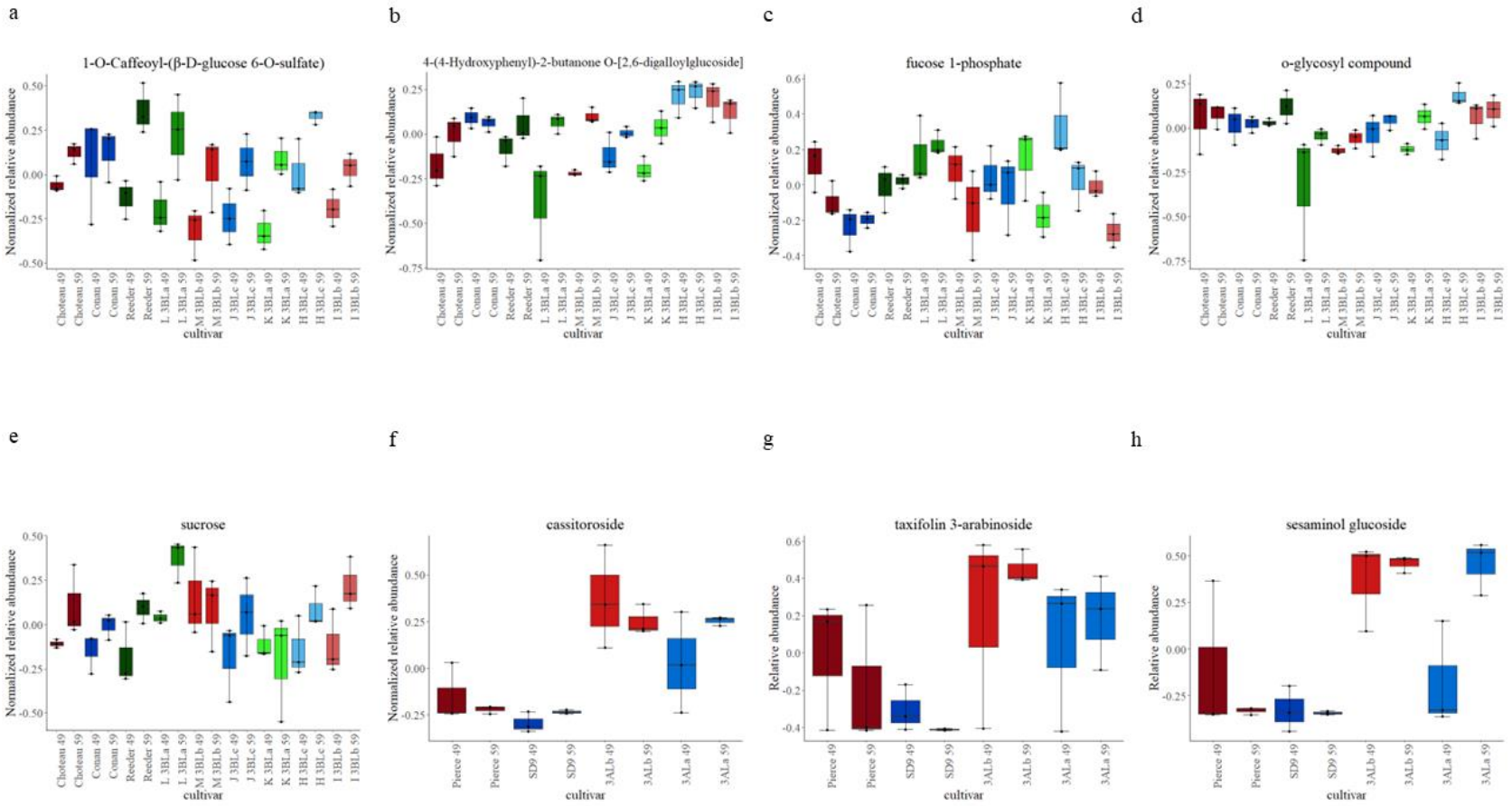


Figure 3.10. Relative abundance of phospholipids and related metabolites in spring and durum wheat

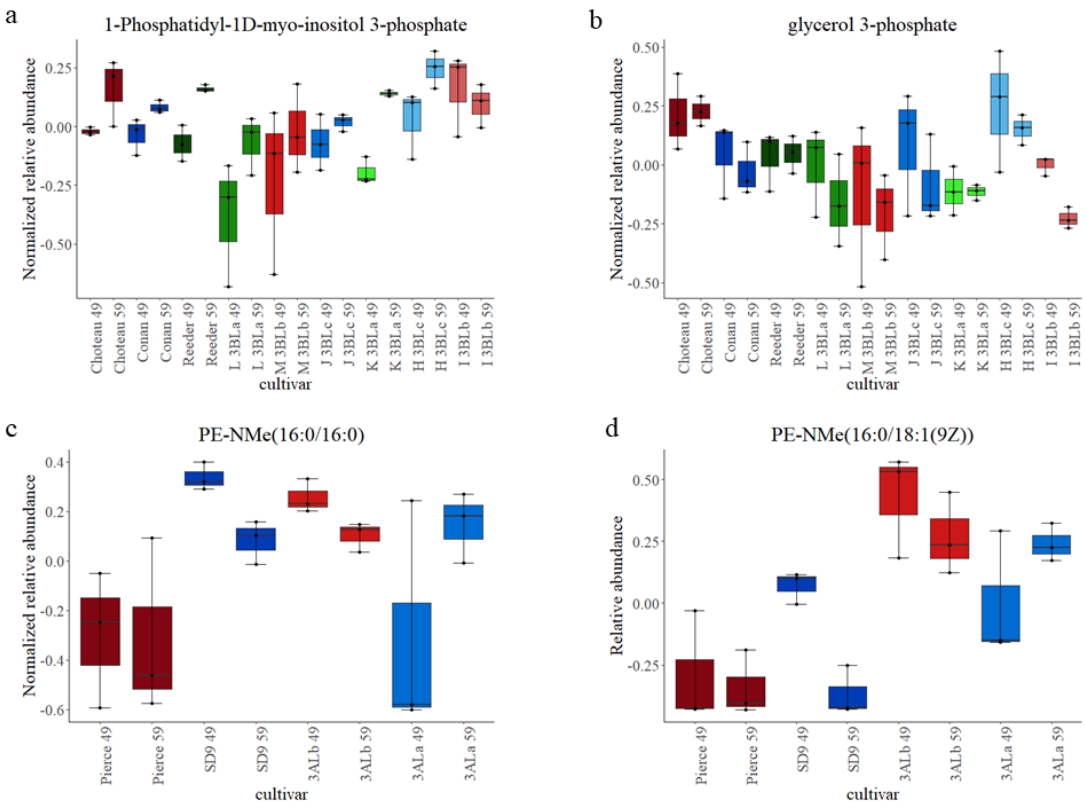


Figure 3.11 Principal component analysis of metabolites from spring wheat near isogenic lines with the *3BLc* and *3BLb* alleles

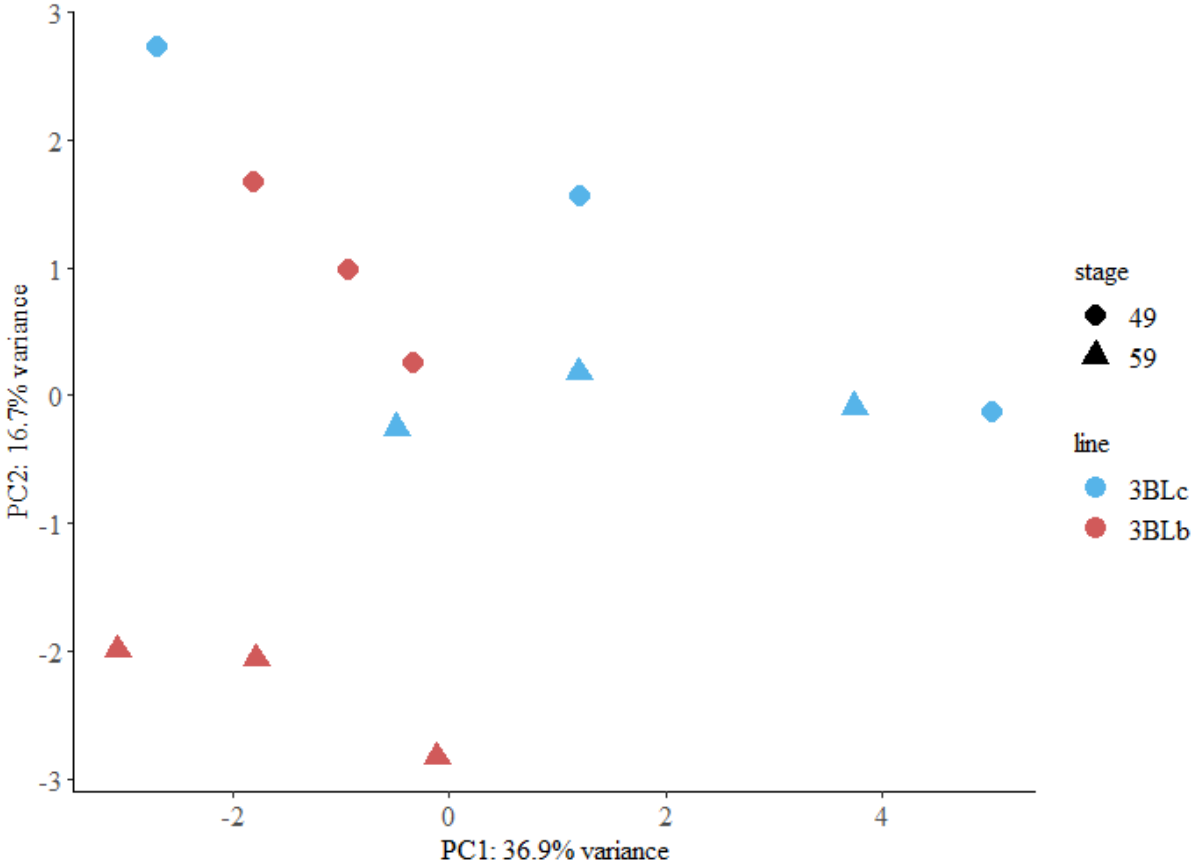


Figure 3.12 Principal component analysis of metabolites in spring wheat near isogenic lines with *3BLc* and *3BLa* alleles

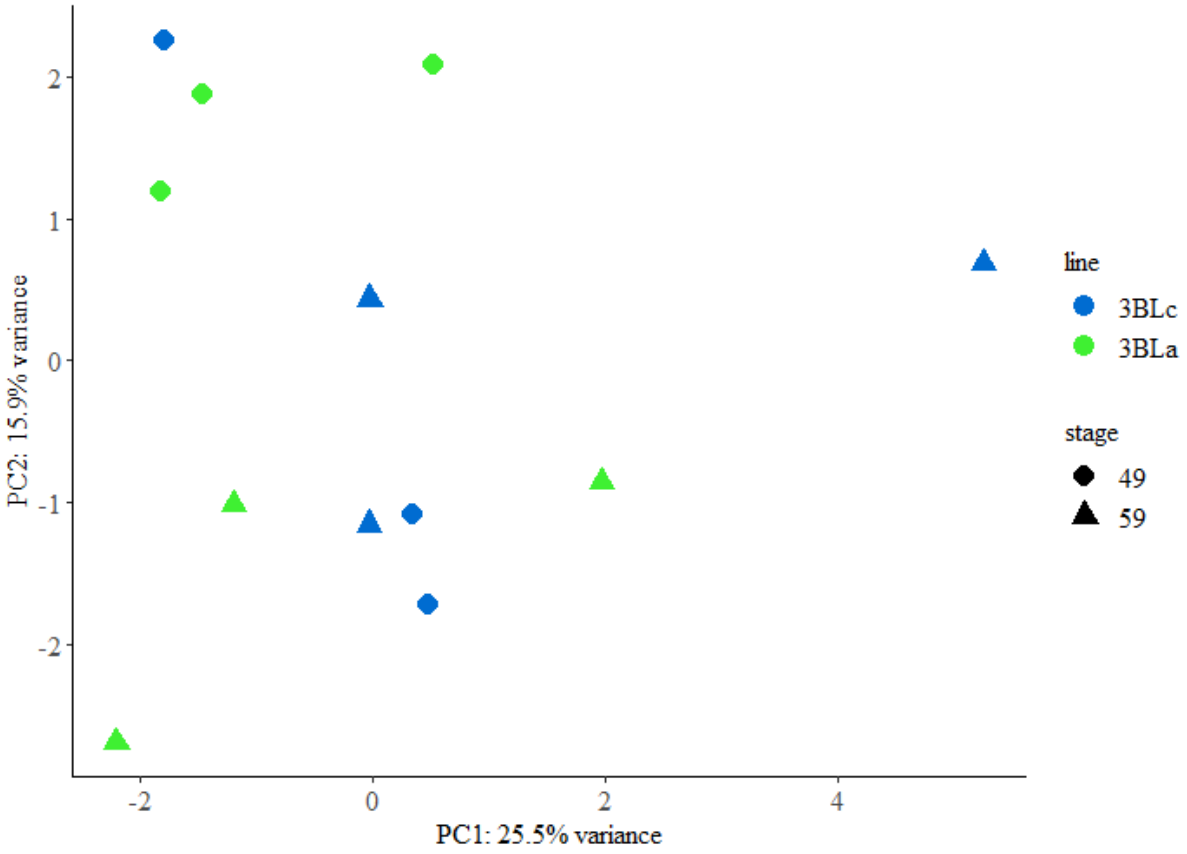


Figure 3.13. Principal component analysis of metabolites in spring wheat near isogenic lines with *3BLa* and *3BLb* alleles

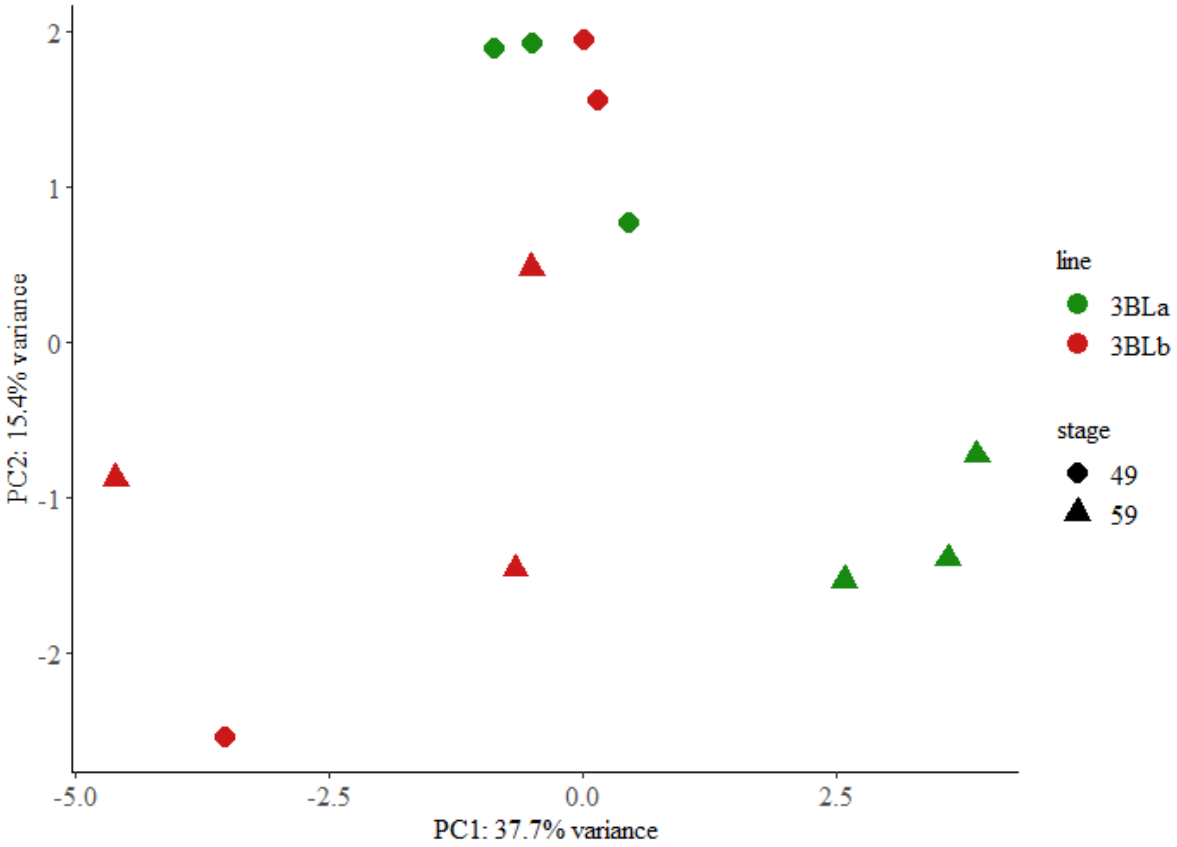


Figure 3.14 Principal component analysis of transcripts of durum wheat

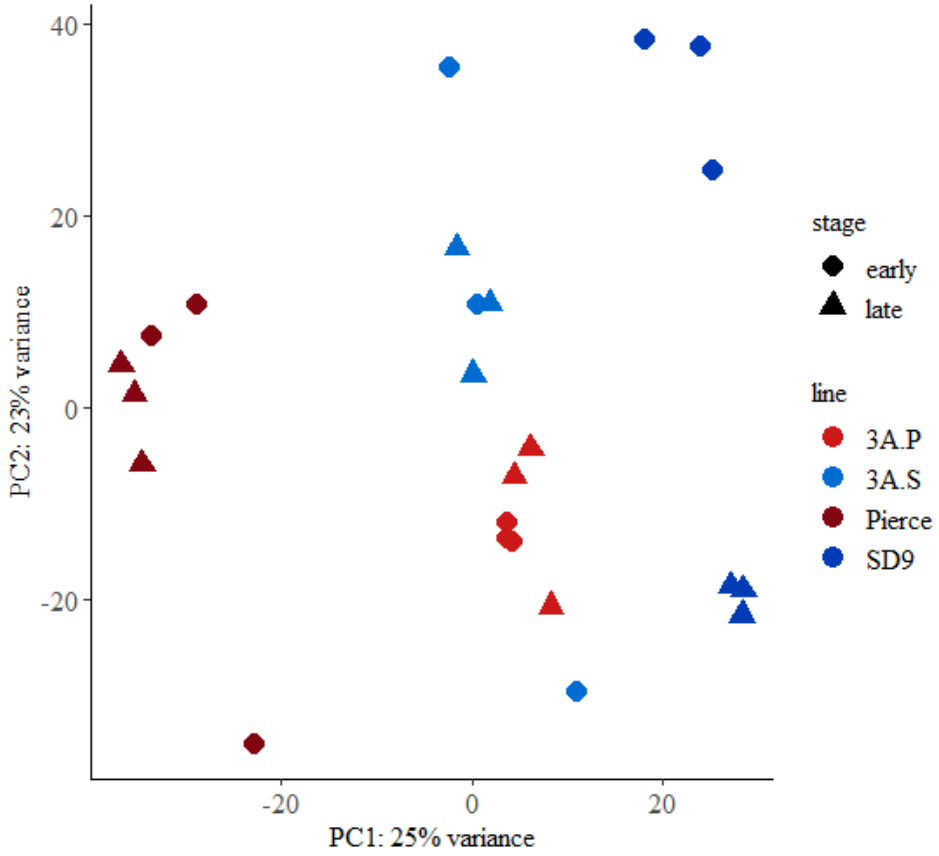


Figure 3.15. Differential expression of durum wheat transcripts with significant allelic differences

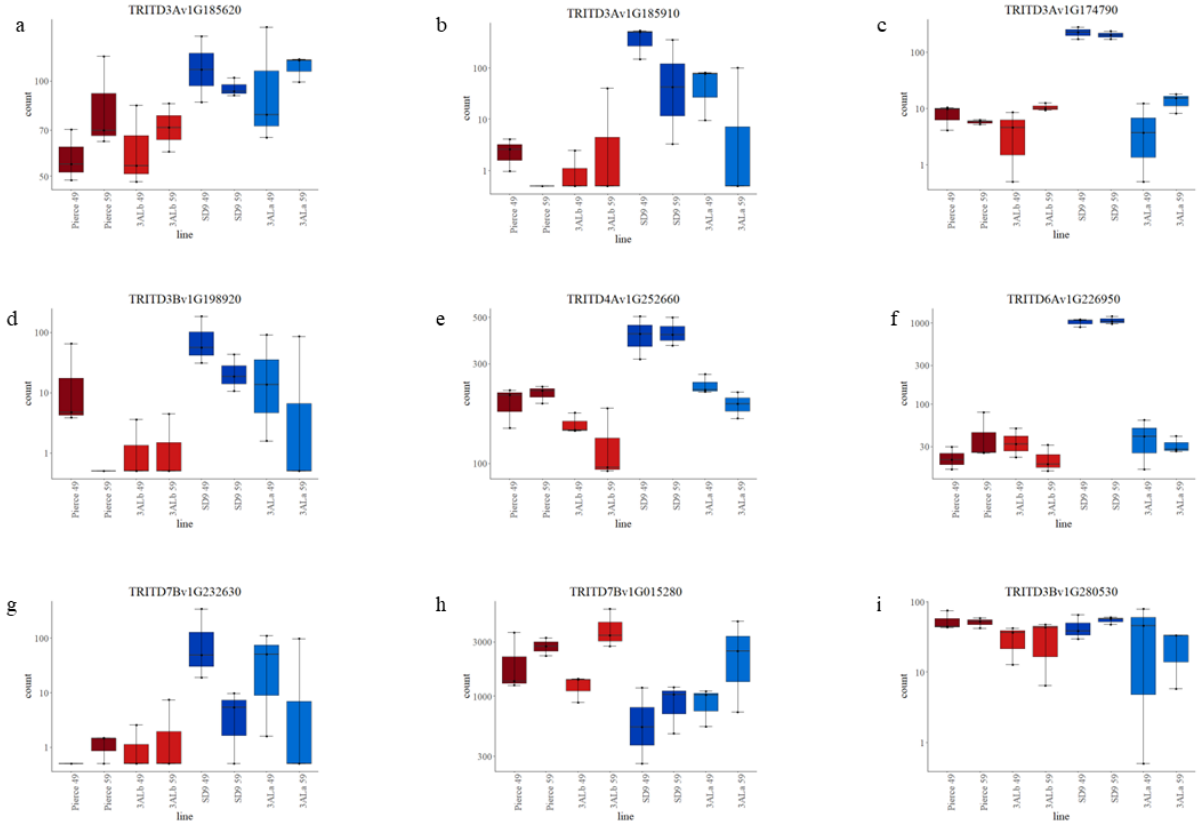


Figure 3.16 Differential expression of durum wheat transcripts with significant growth stage differences

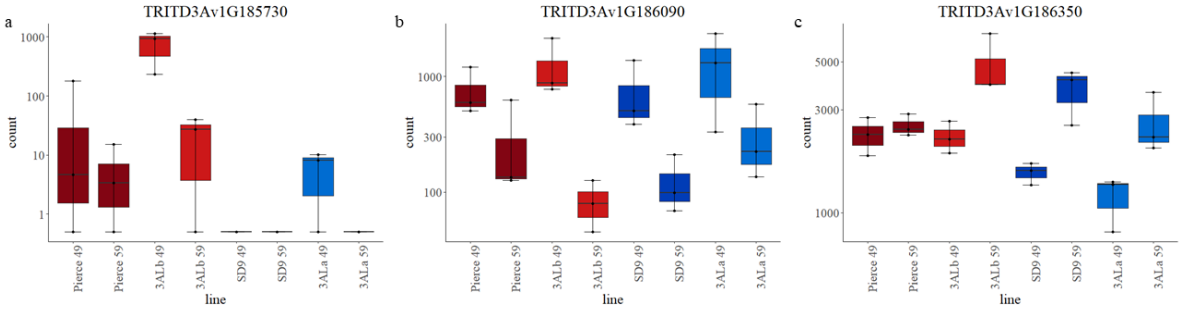


Figure 3.17. Principal component analysis of metabolites in durum wheat

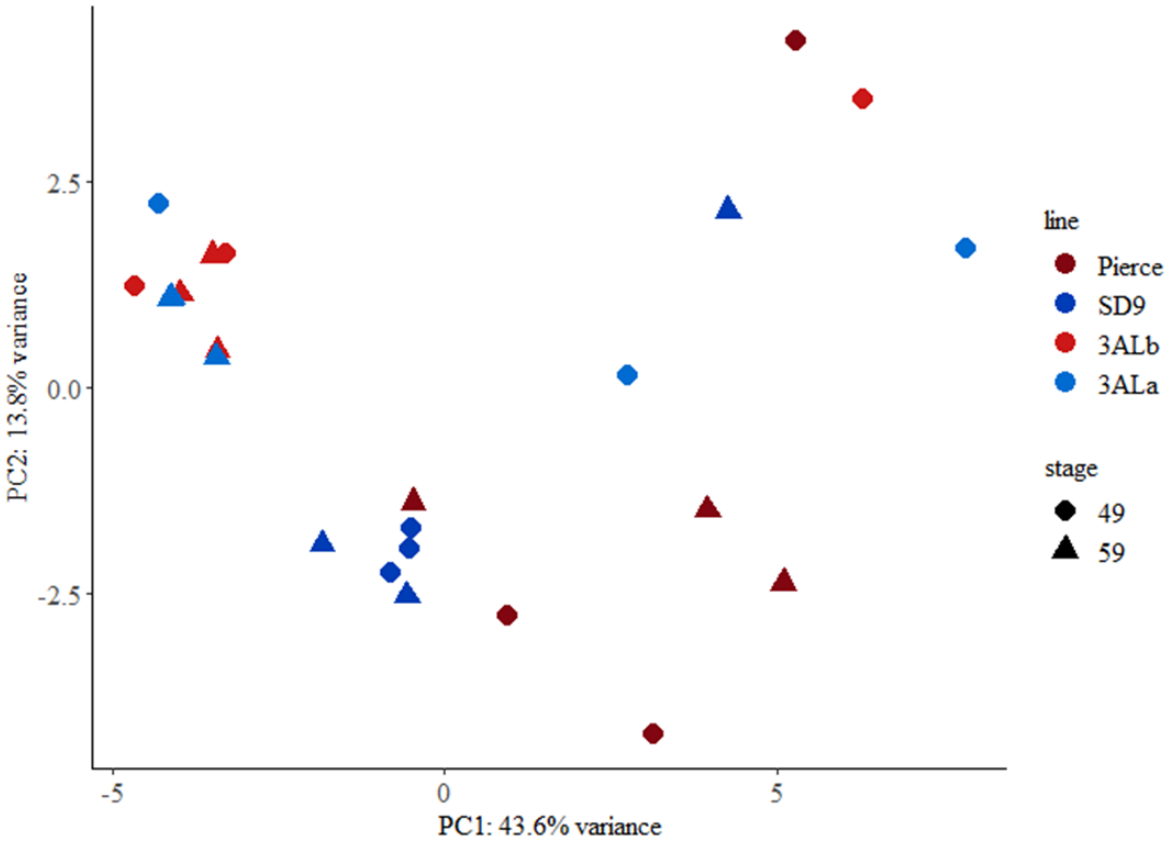
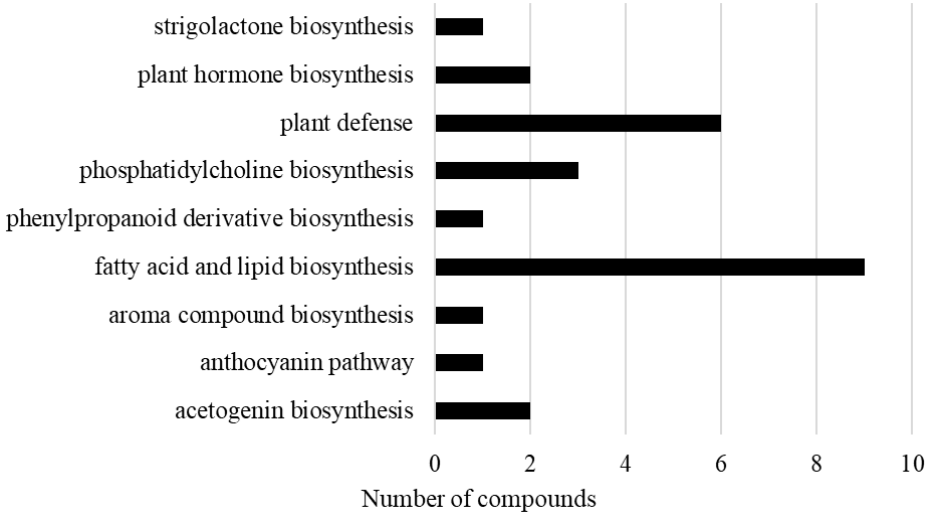


Figure 3.18. Pathway associations and classifications of significant compounds in durum wheat



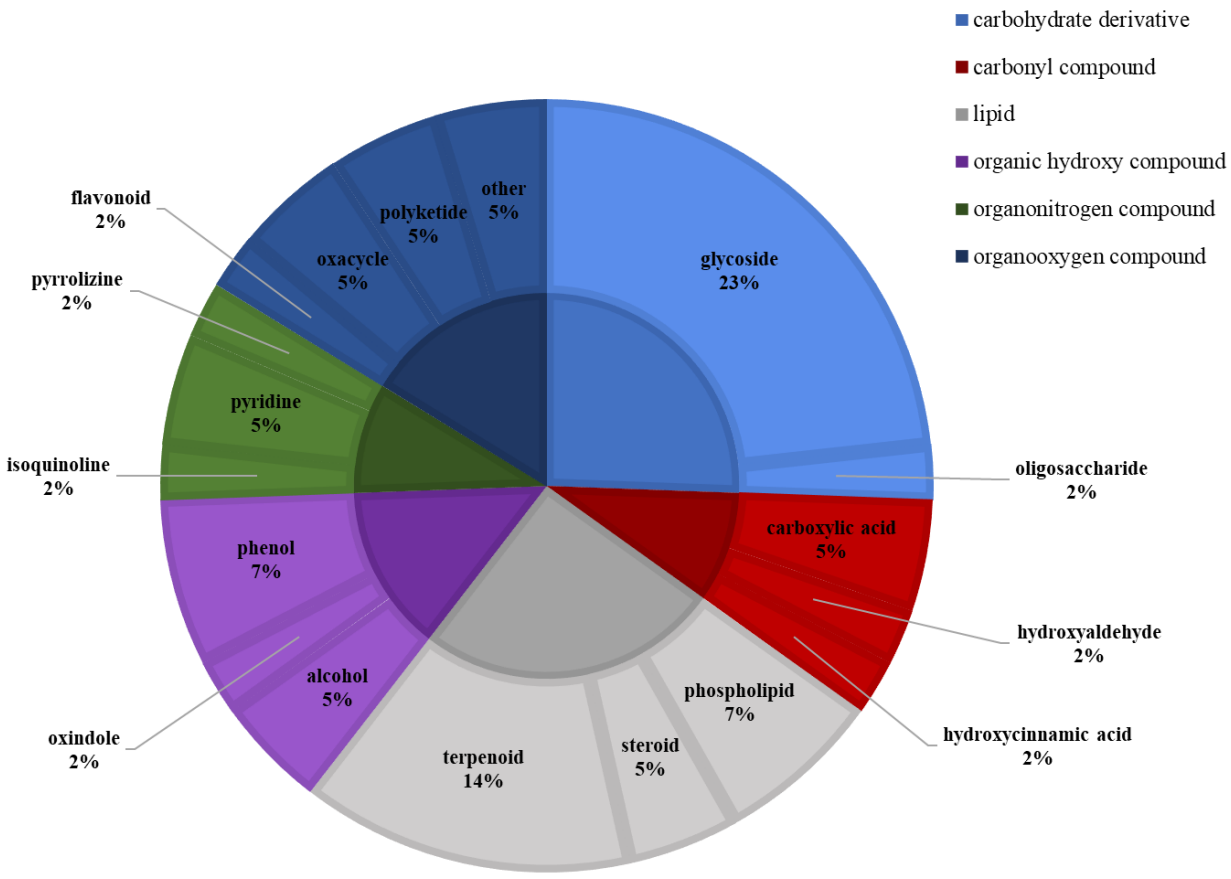


Figure 3.19. Principal component analysis of metabolites in near isogenic lines of durum wheat

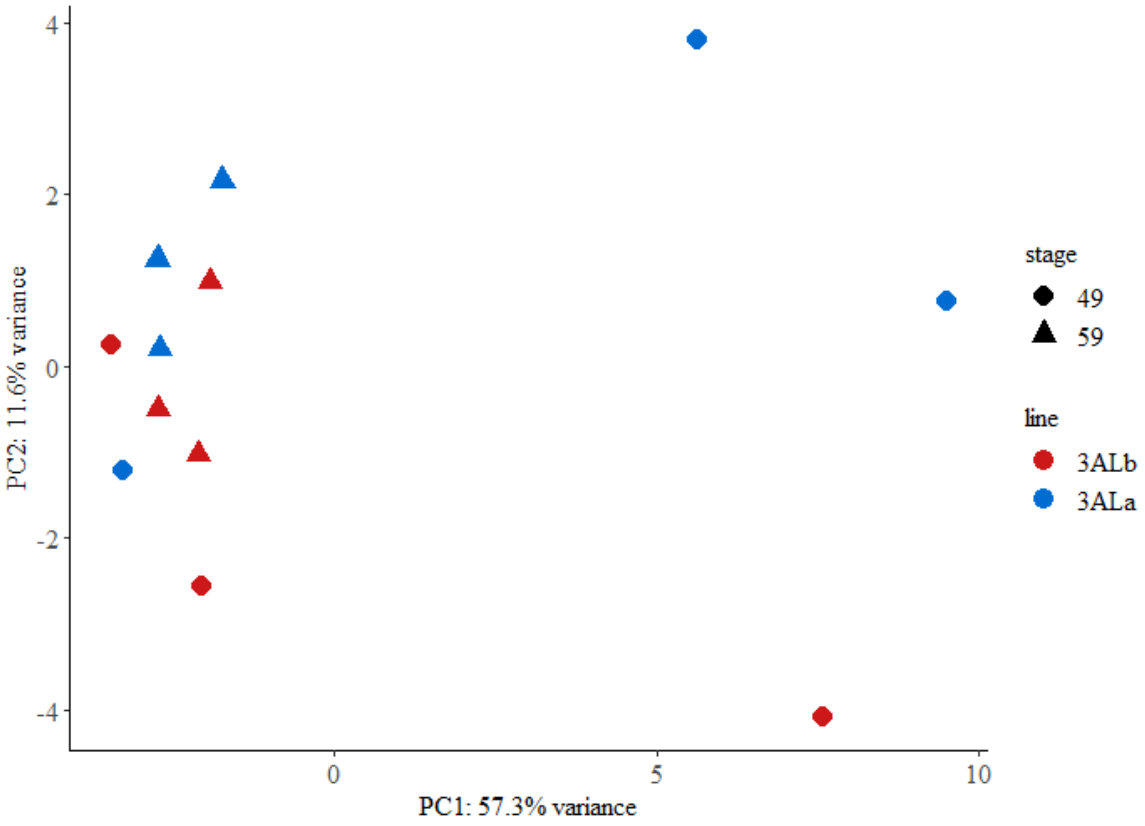


Table 3.1. Transcripts with significant allelic differences in spring wheat

Gene ID	base Mean	log2 Fold Change (LFE)	LFE Std Error	Wald Stat	p-val	adj p-val	chr	Transcription Factor Family
TraesCS3B02G001900	763.495	-0.605	0.159	-3.804	0.000	0.012	3B	NA
TraesCS3B02G006600	698.503	2.163	0.646	3.351	0.001	0.034	3B	p450
TraesCS3B02G017800	69.966	1.352	0.408	3.315	0.001	0.036	3B	NA
TraesCS3B02G023200	261.072	-0.982	0.284	-3.463	0.001	0.026	3B	NA
TraesCS3B02G039700	425.020	-1.229	0.256	-4.796	0.000	0.001	3B	NA
TraesCS3B02G042400	348.221	1.809	0.460	3.933	0.000	0.009	3B	NA
TraesCS3B02G048800	1061.395	1.269	0.396	3.208	0.001	0.044	3B	NA
TraesCS3B02G062700	1125.160	1.999	0.419	4.773	0.000	0.001	3B	NA
TraesCS3B02G063400	88.465	2.298	0.503	4.573	0.000	0.001	3B	NA
TraesCS3B02G072500	300.539	-1.750	0.321	-5.451	0.000	0.000	3B	NA
TraesCS3B02G077400	80.180	0.584	0.166	3.510	0.000	0.024	3B	NA
TraesCS3B02G099300	58.598	2.571	0.731	3.519	0.000	0.023	3B	NA
TraesCS3B02G101700	212.470	1.321	0.283	4.674	0.000	0.001	3B	NB-ARC
TraesCS3B02G102900	38.569	1.093	0.315	3.477	0.001	0.025	3B	NA
TraesCS3B02G108100	807.775	-0.481	0.142	-3.392	0.001	0.031	3B	NA
TraesCS3B02G112000	1577.902	0.901	0.271	3.325	0.001	0.035	3B	NA
TraesCS3B02G119400	49.989	2.239	0.685	3.268	0.001	0.039	3B	NA
TraesCS3B02G125600	138.957	1.208	0.304	3.973	0.000	0.008	3B	zf-CCCH
TraesCS3B02G131500	1316.400	0.294	0.091	3.229	0.001	0.042	3B	UQ_con
TraesCS3B02G142800	294.435	-0.693	0.212	-3.262	0.001	0.039	3B	NA
TraesCS3B02G148400	111.115	0.889	0.242	3.674	0.000	0.016	3B	NA
TraesCS3B02G154300	35.385	2.771	0.837	3.312	0.001	0.036	3B	ECH
TraesCS3B02G156900	619.031	0.780	0.237	3.294	0.001	0.037	3B	zf-B_box
TraesCS3B02G157400	362.745	0.725	0.195	3.706	0.000	0.015	3B	NA

Table 3.1 continued

TraesCS3B02G158200	100.299	-1.073	0.293	-3.659	0.000	0.017	3B	NA
TraesCS3B02G164700	193.370	1.580	0.458	3.450	0.001	0.027	3B	NA
TraesCS3B02G167800	265.595	-0.568	0.171	-3.324	0.001	0.035	3B	Glyco_transf_22
TraesCS3B02G177700	510.794	-0.587	0.185	-3.172	0.002	0.047	3B	Sybindin
TraesCS3B02G181500	4353.465	-0.590	0.166	-3.553	0.000	0.021	3B	NA
TraesCS3B02G209300	146.129	-1.158	0.361	-3.210	0.001	0.044	3B	Glycos_transf_1
TraesCS3B02G222700	560.525	0.588	0.186	3.161	0.002	0.048	3B	NA
TraesCS3B02G229000	597.584	0.998	0.312	3.198	0.001	0.045	3B	NA
TraesCS3B02G236900	331.179	1.279	0.397	3.224	0.001	0.043	3B	HLH
TraesCS3B02G248500	411.057	2.479	0.597	4.154	0.000	0.005	3B	ABC2_membrane
TraesCS3B02G253500	71.496	2.420	0.726	3.333	0.001	0.035	3B	NA
TraesCS3B02G255300	2797.710	-0.501	0.125	-4.000	0.000	0.007	3B	NA
TraesCS3B02G261800	419.701	-3.736	1.105	-3.381	0.001	0.032	3B	zf-CCCH
TraesCS3B02G265700	6439.165	0.749	0.208	3.598	0.000	0.020	3B	NA
TraesCS3B02G267000	302.145	-0.450	0.140	-3.207	0.001	0.044	3B	NA
TraesCS3B02G268700	264.994	0.582	0.185	3.145	0.002	0.050	3B	ECH
TraesCS3B02G275200	5367.280	0.564	0.166	3.403	0.001	0.030	3B	NA
TraesCS3B02G294200	364.038	-0.606	0.142	-4.274	0.000	0.004	3B	Metallophos
TraesCS3B02G308700	497.605	-0.613	0.192	-3.185	0.001	0.046	3B	NA
TraesCS3B02G310300	537.852	1.237	0.286	4.320	0.000	0.003	3B	NAD_binding_2
TraesCS3B02G318600	88.766	1.108	0.349	3.175	0.001	0.047	3B	NA
TraesCS3B02G321600	170.323	0.669	0.209	3.199	0.001	0.045	3B	Hexapep
TraesCS3B02G330700	102.008	-0.539	0.140	-3.856	0.000	0.011	3B	NA
TraesCS3B02G339100	30.593	-1.045	0.292	-3.580	0.000	0.020	3B	Peptidase_S8
TraesCS3B02G339200	277.163	0.759	0.190	3.998	0.000	0.007	3B	NA
TraesCS3B02G365400	3739.949	-0.447	0.114	-3.919	0.000	0.009	3B	LRRNT_2
TraesCS3B02G365900	1068.539	-0.502	0.148	-3.402	0.001	0.030	3B	NA
TraesCS3B02G377300	83.345	2.787	0.821	3.396	0.001	0.030	3B	NA

Table 3.1 continued

TraesCS3B02G388900	550.082	-0.418	0.121	-3.459	0.001	0.027	3B	Ras
TraesCS3B02G395400	974.536	-0.337	0.101	-3.346	0.001	0.034	3B	Hexapep
TraesCS3B02G396900	408.668	0.480	0.120	4.003	0.000	0.007	3B	NA
TraesCS3B02G399100	196.243	1.055	0.332	3.175	0.001	0.047	3B	NA
TraesCS3B02G415900	260.166	1.006	0.274	3.674	0.000	0.016	3B	Pribosyltran
TraesCS3B02G422500	158.153	0.947	0.281	3.371	0.001	0.032	3B	NA
TraesCS3B02G422700	662.653	-0.730	0.183	-3.983	0.000	0.008	3B	NA
TraesCS3B02G437300	371.762	0.513	0.131	3.917	0.000	0.009	3B	NA
TraesCS3B02G448000	503.700	1.621	0.380	4.271	0.000	0.004	3B	zf-TAZ
TraesCS3B02G452600	86.547	-1.314	0.345	-3.810	0.000	0.012	3B	NA
TraesCS3B02G458700	188.259	-0.858	0.237	-3.613	0.000	0.019	3B	NA
TraesCS3B02G472600	278.989	-1.151	0.271	-4.253	0.000	0.004	3B	NA
TraesCS3B02G487800	346.575	-0.408	0.128	-3.192	0.001	0.046	3B	NA
TraesCS3B02G505400	104.124	-0.885	0.279	-3.172	0.002	0.047	3B	NA
TraesCS3B02G506700	1094.057	-1.802	0.493	-3.654	0.000	0.017	3B	Lipase_3
TraesCS3B02G511900	750.060	-0.447	0.095	-4.702	0.000	0.001	3B	Motile_Sperm
TraesCS3B02G520100	2085.980	0.819	0.192	4.275	0.000	0.004	3B	2OG-FeII_Oxy
TraesCS3B02G530200	157.796	1.307	0.366	3.569	0.000	0.021	3B	B3
TraesCS3B02G536600	802.263	0.958	0.281	3.402	0.001	0.030	3B	NA
TraesCS3B02G545500	1089.358	-0.373	0.118	-3.162	0.002	0.048	3B	NA
TraesCS3B02G559400	546.947	-0.548	0.136	-4.036	0.000	0.007	3B	NA
TraesCS3B02G563800	47.697	2.536	0.629	4.030	0.000	0.007	3B	p450
TraesCS3B02G566100	245.408	-1.301	0.357	-3.648	0.000	0.017	3B	NA
TraesCS3B02G571000	333.812	-0.609	0.190	-3.216	0.001	0.043	3B	NA
TraesCS3B02G573400	180.820	4.997	0.542	9.217	0.000	0.000	3B	NB-ARC
TraesCS3B02G573500	36.919	8.549	0.650	13.154	0.000	0.000	3B	NB-ARC
TraesCS3B02G573600	116.247	10.237	0.606	16.906	0.000	0.000	3B	NA
TraesCS3B02G573700	55.237	7.712	0.648	11.896	0.000	0.000	3B	NB-ARC

Table 3.1 continued

TraesCS3B02G575600	297.733	2.079	0.182	11.399	0.000	0.000	3B	NA
TraesCS3B02G576100	28.303	2.988	0.499	5.983	0.000	0.000	3B	NA
TraesCS3B02G577800	167.968	1.500	0.237	6.325	0.000	0.000	3B	NA
TraesCS3B02G579700	37.749	3.758	0.635	5.919	0.000	0.000	3B	NA
TraesCS3B02G581300	719.842	0.731	0.102	7.155	0.000	0.000	3B	NA
TraesCS3B02G581900	226.895	4.011	0.275	14.609	0.000	0.000	3B	AA_kinase
TraesCS3B02G582500	291.345	-0.429	0.134	-3.211	0.001	0.044	3B	NA
TraesCS3B02G583100	57.528	7.474	0.595	12.554	0.000	0.000	3B	NA
TraesCS3B02G585600	90.502	3.007	0.624	4.817	0.000	0.001	3B	Peptidase_M18
TraesCS3B02G585700	178.182	4.766	0.447	10.660	0.000	0.000	3B	NA
TraesCS3B02G587400	39.165	2.195	0.471	4.655	0.000	0.001	3B	NA
TraesCS3B02G587700	69.078	3.485	0.475	7.335	0.000	0.000	3B	BACK
TraesCS3B02G588000	34.456	-2.242	0.367	-6.112	0.000	0.000	3B	NA
TraesCS3B02G588500	42.962	6.964	0.660	10.558	0.000	0.000	3B	NB-ARC
TraesCS3B02G589700	87.785	4.638	0.567	8.181	0.000	0.000	3B	NB-ARC
TraesCS3B02G589900	216.673	1.287	0.210	6.124	0.000	0.000	3B	NA
TraesCS3B02G590000	696.223	-1.052	0.286	-3.677	0.000	0.016	3B	Epimerase
TraesCS3B02G591400	272.248	-0.547	0.166	-3.289	0.001	0.037	3B	NA
TraesCS3B02G591800	46.819	8.920	0.627	14.216	0.000	0.000	3B	NA
TraesCS3B02G593200	101.201	2.499	0.347	7.202	0.000	0.000	3B	NA
TraesCS3B02G593300	43.608	8.787	0.708	12.409	0.000	0.000	3B	NA
TraesCS3B02G593900	76.788	2.755	0.597	4.613	0.000	0.001	3B	NB-ARC
TraesCS3B02G594300	118.375	6.907	0.590	11.712	0.000	0.000	3B	Myb_DNA-binding
TraesCS3B02G594700	106.266	-5.106	0.678	-7.527	0.000	0.000	3B	NA
TraesCS3B02G596400	73.316	-5.924	0.820	-7.220	0.000	0.000	3B	p450
TraesCS3B02G596800	121.646	-3.883	0.906	-4.285	0.000	0.003	3B	p450
TraesCS3B02G597000	143.464	-4.461	0.934	-4.776	0.000	0.001	3B	p450

Table 3.1 continued

TraesCS3B02G597800	12884.506	-1.571	0.456	-3.444	0.001	0.027	3B	NA
TraesCS3B02G597900	2903.821	1.753	0.393	4.464	0.000	0.002	3B	NA
TraesCS3B02G598200	45.780	4.394	0.582	7.554	0.000	0.000	3B	UDPGT
TraesCS3B02G599600	81.775	-1.324	0.218	-6.078	0.000	0.000	3B	NA
TraesCS3B02G601600	141.664	1.702	0.449	3.794	0.000	0.012	3B	NA
TraesCS3B02G602500	82.247	-2.978	0.656	-4.540	0.000	0.001	3B	Lipoxygenase
TraesCS3B02G602700	655.602	0.920	0.251	3.666	0.000	0.017	3B	Pkinase
TraesCS3B02G603900	71.278	5.884	0.637	9.231	0.000	0.000	3B	GATA
TraesCS3B02G608500	445.366	1.930	0.544	3.549	0.000	0.022	3B	NA
TraesCS3B02G610100	62.453	3.505	0.787	4.450	0.000	0.002	3B	NA
TraesCS3B02G612000	13664.405	2.300	0.512	4.490	0.000	0.002	3B	NA

Table 3.2. Transcripts with significant growth stage differences in spring wheat

Gene ID	**base Mean**	**log2 Fold Change (LFC)**	**LFC Std Error**	**Wald stat**	**p-val**	**adj p-val**	**chr**	**Transcription Factor Family**
TraesCS3B02G004600	792.798	1.288	0.270	4.765	0.000	0.000	3B	Peptidase_S8
TraesCS3B02G005800	511.240	-0.688	0.176	-3.904	0.000	0.005	3B	NA
TraesCS3B02G006300	409.661	-1.027	0.336	-3.060	0.002	0.039	3B	NA
TraesCS3B02G020700	204.640	1.040	0.351	2.960	0.003	0.049	3B	NA
TraesCS3B02G021700	71.787	-2.311	0.686	-3.367	0.001	0.019	3B	NA
TraesCS3B02G022000	56.897	-2.933	0.638	-4.599	0.000	0.001	3B	NA
TraesCS3B02G024900	595.227	0.467	0.137	3.407	0.001	0.018	3B	HA2
TraesCS3B02G025600	245.101	3.797	0.746	5.091	0.000	0.000	3B	p450
TraesCS3B02G032900	29.597	-2.514	0.685	-3.668	0.000	0.009	3B	NA
TraesCS3B02G040800	435.076	-0.851	0.277	-3.069	0.002	0.038	3B	NA
TraesCS3B02G049900	280.533	-2.309	0.742	-3.111	0.002	0.035	3B	NA
TraesCS3B02G059700	95.671	-2.178	0.737	-2.957	0.003	0.049	3B	MR_MLE_N

Table 3.2 continued

TraesCS3B02G064700	278.188	-4.043	0.780	-5.183	0.000	0.000	3B	NA
TraesCS3B02G064800	122.546	-1.763	0.436	-4.042	0.000	0.003	3B	NA
TraesCS3B02G064900	65.273	-2.439	0.749	-3.259	0.001	0.025	3B	B3
TraesCS3B02G068800	290.984	0.631	0.184	3.435	0.001	0.017	3B	Myb_DNA-binding
TraesCS3B02G068900	1751.576	0.495	0.149	3.330	0.001	0.021	3B	Myb_DNA-binding
TraesCS3B02G098100	54.968	-1.329	0.447	-2.972	0.003	0.048	3B	Phospho esterase
TraesCS3B02G106900	460.684	-2.138	0.644	-3.320	0.001	0.022	3B	NA
TraesCS3B02G108700	280.290	-4.831	0.844	-5.723	0.000	0.000	3B	NA
TraesCS3B02G112800	1275.859	-0.594	0.184	-3.227	0.001	0.027	3B	NA
TraesCS3B02G113500	760.223	-0.719	0.220	-3.265	0.001	0.025	3B	NA
TraesCS3B02G120700	5818.736	-1.879	0.550	-3.416	0.001	0.017	3B	NA
TraesCS3B02G121000	315.935	0.771	0.221	3.493	0.000	0.014	3B	Ribonuclease_3
TraesCS3B02G125600	138.957	-1.253	0.304	-4.120	0.000	0.003	3B	zf-CCCH
TraesCS3B02G128000	1150.527	-1.142	0.353	-3.236	0.001	0.026	3B	Malic_M
TraesCS3B02G129200	26.288	-1.555	0.448	-3.471	0.001	0.015	3B	NA
TraesCS3B02G132400	2501.485	-1.204	0.301	-3.999	0.000	0.004	3B	NA
TraesCS3B02G135500	139.584	-1.974	0.523	-3.773	0.000	0.007	3B	NA
TraesCS3B02G136000	62.930	-2.991	0.549	-5.449	0.000	0.000	3B	NA
TraesCS3B02G140700	1440.880	-0.926	0.253	-3.667	0.000	0.009	3B	NA
TraesCS3B02G140800	107.643	-1.402	0.310	-4.518	0.000	0.001	3B	NA
TraesCS3B02G141700	949.587	-1.216	0.358	-3.397	0.001	0.018	3B	ABC2_ membrane
TraesCS3B02G141800	251.482	-3.290	0.801	-4.106	0.000	0.003	3B	2OG-FeII_Oxy
TraesCS3B02G148400	111.115	0.850	0.242	3.513	0.000	0.014	3B	NA

Table 3.2 continued

TraesCS3B02G152300	934.706	-1.554	0.488	-3.181	0.001	0.030	3B	GST_N
TraesCS3B02G153700	297.307	0.393	0.131	3.006	0.003	0.044	3B	NA
TraesCS3B02G154900	163.291	1.287	0.336	3.828	0.000	0.006	3B	PPR
TraesCS3B02G158500	312.358	1.026	0.296	3.468	0.001	0.015	3B	NA
TraesCS3B02G162100	61.517	-2.541	0.834	-3.047	0.002	0.040	3B	NA
TraesCS3B02G162500	86.034	-0.931	0.301	-3.092	0.002	0.036	3B	Pkinase_Tyr
TraesCS3B02G163100	290.582	-2.270	0.625	-3.630	0.000	0.010	3B	NA
TraesCS3B02G163300	128.166	-5.533	0.927	-5.967	0.000	0.000	3B	Lipase_GDSL
TraesCS3B02G163700	617.701	-1.557	0.402	-3.876	0.000	0.005	3B	Lipase_GDSL
TraesCS3B02G168300	566.330	-1.229	0.368	-3.343	0.001	0.021	3B	NA
TraesCS3B02G170500	125.488	-0.814	0.156	-5.234	0.000	0.000	3B	NA
TraesCS3B02G173900	92.055	-1.834	0.582	-3.149	0.002	0.032	3B	NA
TraesCS3B02G176400	27.878	1.996	0.576	3.467	0.001	0.015	3B	NA
TraesCS3B02G182400	67.669	4.550	0.735	6.194	0.000	0.000	3B	NA
TraesCS3B02G191600	1554.866	-0.738	0.182	-4.060	0.000	0.003	3B	Myb_DNA-binding
TraesCS3B02G199100	127.655	-1.233	0.340	-3.629	0.000	0.010	3B	Pkinase
TraesCS3B02G203100	1887.615	0.807	0.219	3.679	0.000	0.009	3B	NA
TraesCS3B02G204300	393.034	-0.815	0.225	-3.622	0.000	0.010	3B	NA
TraesCS3B02G205100	782.179	-3.104	0.678	-4.582	0.000	0.001	3B	NA
TraesCS3B02G207700	218.387	-1.256	0.369	-3.400	0.001	0.018	3B	NA
TraesCS3B02G209900	483.539	-3.006	1.002	-2.999	0.003	0.045	3B	NA
TraesCS3B02G212400	185.981	-2.609	0.462	-5.642	0.000	0.000	3B	NA
TraesCS3B02G219800	560.826	0.369	0.094	3.942	0.000	0.004	3B	NA
TraesCS3B02G220600	237.010	1.076	0.320	3.357	0.001	0.020	3B	NA
TraesCS3B02G220900	340.565	-0.561	0.174	-3.229	0.001	0.027	3B	NA
TraesCS3B02G224500	259.558	-2.159	0.526	-4.104	0.000	0.003	3B	Myb_DNA-binding

Table 3.2 continued

TraesCS3B02G233200	120.909	0.777	0.250	3.109	0.002	0.035	3B	NA
TraesCS3B02G238300	1232.398	-0.993	0.288	-3.449	0.001	0.016	3B	NA
TraesCS3B02G241800	156.385	0.461	0.138	3.332	0.001	0.021	3B	S1
TraesCS3B02G244800	104.922	-2.129	0.581	-3.663	0.000	0.009	3B	NA
TraesCS3B02G251600	75.699	-3.267	0.888	-3.678	0.000	0.009	3B	NA
TraesCS3B02G252800	64.976	-4.138	0.661	-6.257	0.000	0.000	3B	Lipase_3
TraesCS3B02G253000	297.800	-1.851	0.429	-4.311	0.000	0.001	3B	NA
TraesCS3B02G254200	122.251	-1.018	0.262	-3.884	0.000	0.005	3B	Glycos_ transf_1
TraesCS3B02G261800	419.701	-3.263	1.105	-2.953	0.003	0.050	3B	zf-CCCH
TraesCS3B02G262400	46.730	-3.139	0.793	-3.960	0.000	0.004	3B	GRAS
TraesCS3B02G263600	1317.971	-1.087	0.303	-3.584	0.000	0.011	3B	NA
TraesCS3B02G264000	79.041	-4.396	0.872	-5.039	0.000	0.000	3B	NA
TraesCS3B02G269900	118.221	-3.146	0.715	-4.398	0.000	0.001	3B	Pkinase
TraesCS3B02G272600	271.206	-1.002	0.258	-3.883	0.000	0.005	3B	NA
TraesCS3B02G272700	436.650	-4.466	1.050	-4.252	0.000	0.002	3B	NA
TraesCS3B02G278400	23.798	-3.497	1.117	-3.130	0.002	0.034	3B	Glyco_hydro_ 17
TraesCS3B02G284100	65.123	-0.937	0.297	-3.155	0.002	0.032	3B	NA
TraesCS3B02G285700	2384.545	0.947	0.248	3.818	0.000	0.006	3B	NA
TraesCS3B02G287000	644.266	0.347	0.105	3.297	0.001	0.023	3B	CRAL_TRIO _N
TraesCS3B02G297700	91.750	-1.361	0.416	-3.272	0.001	0.024	3B	NA
TraesCS3B02G300300	79.113	1.642	0.528	3.112	0.002	0.035	3B	NA
TraesCS3B02G307400	106.312	0.623	0.187	3.338	0.001	0.021	3B	NA
TraesCS3B02G309500	136.176	-1.697	0.484	-3.506	0.000	0.014	3B	TFR_dimer
TraesCS3B02G309600	84.271	-1.325	0.301	-4.406	0.000	0.001	3B	IQ
TraesCS3B02G310500	54.608	-2.555	0.527	-4.852	0.000	0.000	3B	NA

Table 3.2 continued

TraesCS3B02G311100	28.226	-2.848	0.847	-3.363	0.001	0.020	3B	NA
TraesCS3B02G313900	53.559	-2.734	0.841	-3.249	0.001	0.026	3B	UDPGT
TraesCS3B02G315200	404.626	1.275	0.367	3.473	0.001	0.015	3B	NA
TraesCS3B02G322600	538.731	0.337	0.114	2.964	0.003	0.049	3B	PHD
TraesCS3B02G323900	722.439	-0.982	0.259	-3.787	0.000	0.007	3B	NA
TraesCS3B02G324400	467.136	-0.735	0.228	-3.226	0.001	0.027	3B	WRKY
TraesCS3B02G325700	62.060	1.009	0.321	3.147	0.002	0.032	3B	NA
TraesCS3B02G328900	147.408	-2.512	0.768	-3.273	0.001	0.024	3B	NA
TraesCS3B02G329700	47.907	-2.184	0.496	-4.407	0.000	0.001	3B	zf-Dof
TraesCS3B02G330600	639.756	0.617	0.177	3.498	0.000	0.014	3B	Pkinase
TraesCS3B02G330700	102.008	0.492	0.140	3.518	0.000	0.013	3B	NA
TraesCS3B02G333100	271.974	-3.115	0.997	-3.126	0.002	0.034	3B	NA
TraesCS3B02G335100	122.178	0.789	0.174	4.549	0.000	0.001	3B	AA_kinase
TraesCS3B02G337100	359.457	-2.162	0.499	-4.329	0.000	0.001	3B	NA
TraesCS3B02G339100	30.593	-1.377	0.292	-4.712	0.000	0.000	3B	Peptidase_S8
TraesCS3B02G339200	277.163	0.722	0.190	3.806	0.000	0.006	3B	NA
TraesCS3B02G347300	206.487	-4.774	1.097	-4.351	0.000	0.001	3B	NA
TraesCS3B02G347600	622.793	0.795	0.223	3.566	0.000	0.012	3B	NA
TraesCS3B02G352900	171.950	-2.306	0.522	-4.418	0.000	0.001	3B	Pkinase_Tyr
TraesCS3B02G354100	193.212	2.744	0.689	3.984	0.000	0.004	3B	NA
TraesCS3B02G354800	127.553	-2.146	0.721	-2.978	0.003	0.047	3B	Cu_bind_like
TraesCS3B02G356500	145.887	2.638	0.779	3.387	0.001	0.019	3B	NA
TraesCS3B02G357500	282.635	-1.924	0.450	-4.279	0.000	0.002	3B	AP2
TraesCS3B02G357800	323.602	0.519	0.161	3.226	0.001	0.027	3B	NA
TraesCS3B02G359100	250.996	-4.264	1.132	-3.767	0.000	0.007	3B	NA
TraesCS3B02G361600	1781.155	-0.459	0.147	-3.111	0.002	0.035	3B	NA
TraesCS3B02G361800	78.399	0.950	0.295	3.221	0.001	0.027	3B	NA
TraesCS3B02G363400	458.406	-0.396	0.121	-3.264	0.001	0.025	3B	NA

Table 3.2 continued

TraesCS3B02G366700	75.930	-1.216	0.327	-3.715	0.000	0.008	3B	NA
TraesCS3B02G368300	173.467	-0.882	0.237	-3.723	0.000	0.008	3B	bZIP_1
TraesCS3B02G369600	73.634	-2.248	0.712	-3.157	0.002	0.032	3B	NA
TraesCS3B02G370800	268.600	0.516	0.171	3.020	0.003	0.043	3B	NA
TraesCS3B02G373500	1785.037	0.662	0.203	3.267	0.001	0.024	3B	NA
TraesCS3B02G374400	63.860	-2.458	0.760	-3.235	0.001	0.027	3B	Pkinase_Tyr
TraesCS3B02G377600	255.625	-1.626	0.392	-4.148	0.000	0.002	3B	U-box
TraesCS3B02G378000	321.183	-0.470	0.152	-3.101	0.002	0.036	3B	PP2C
TraesCS3B02G378600	538.993	2.140	0.638	3.351	0.001	0.020	3B	Peptidase_C1
TraesCS3B02G379200	339.818	-1.910	0.550	-3.473	0.001	0.015	3B	WRKY
TraesCS3B02G386200	177.047	-3.416	0.864	-3.952	0.000	0.004	3B	NA
TraesCS3B02G386300	317.744	-3.366	1.138	-2.957	0.003	0.049	3B	NA
TraesCS3B02G387900	268.012	1.576	0.412	3.821	0.000	0.006	3B	NA
TraesCS3B02G388300	148.492	2.945	0.857	3.437	0.001	0.016	3B	NA
TraesCS3B02G390500	66.267	-1.692	0.506	-3.347	0.001	0.020	3B	Thaumatin
TraesCS3B02G397600	297.546	0.698	0.180	3.877	0.000	0.005	3B	NA
TraesCS3B02G397800	175.305	-1.220	0.295	-4.128	0.000	0.002	3B	NA
TraesCS3B02G403400	449.172	-2.096	0.591	-3.549	0.000	0.012	3B	p450
TraesCS3B02G409300	1057.973	-1.247	0.334	-3.732	0.000	0.008	3B	NA
TraesCS3B02G418500	329.743	-2.181	0.489	-4.461	0.000	0.001	3B	DnaJ_C
TraesCS3B02G422800	223.085	-1.256	0.424	-2.963	0.003	0.049	3B	NA
TraesCS3B02G422900	5716.323	-1.043	0.271	-3.841	0.000	0.006	3B	NA
TraesCS3B02G426400	41.141	-1.636	0.521	-3.138	0.002	0.033	3B	NA
TraesCS3B02G430200	115.046	-2.922	0.646	-4.522	0.000	0.001	3B	Ribonuclease_T2
TraesCS3B02G441800	412.722	-2.969	0.588	-5.053	0.000	0.000	3B	NA
TraesCS3B02G442700	405.355	-1.593	0.392	-4.059	0.000	0.003	3B	Glyco_transf_8

Table 3.2 continued

TraesCS3B02G448800	550.745	0.376	0.116	3.252	0.001	0.025	3B	NA
TraesCS3B02G449200	361.101	-1.110	0.369	-3.006	0.003	0.044	3B	NA
TraesCS3B02G454800	44.450	-1.534	0.519	-2.954	0.003	0.049	3B	NA
TraesCS3B02G457600	486.820	1.433	0.421	3.402	0.001	0.018	3B	NA
TraesCS3B02G458700	188.259	-0.719	0.237	-3.028	0.002	0.042	3B	NA
TraesCS3B02G468400	143.330	-1.070	0.307	-3.489	0.000	0.014	3B	SBP
TraesCS3B02G470500	91.410	-2.833	0.662	-4.276	0.000	0.002	3B	NA
TraesCS3B02G481000	79.867	-3.497	0.901	-3.879	0.000	0.005	3B	Pkinase
TraesCS3B02G484000	53.399	2.309	0.758	3.046	0.002	0.040	3B	Glyco_hydro_1
TraesCS3B02G497200	112.322	2.390	0.548	4.360	0.000	0.001	3B	NA
TraesCS3B02G517100	170.464	-3.748	0.737	-5.087	0.000	0.000	3B	RRM_1
TraesCS3B02G533100	465.963	-0.970	0.315	-3.078	0.002	0.038	3B	NAM
TraesCS3B02G557300	63.399	0.979	0.311	3.144	0.002	0.033	3B	NB-ARC
TraesCS3B02G563500	37.114	-3.281	0.940	-3.491	0.000	0.014	3B	MatE
TraesCS3B02G581900	226.895	0.905	0.274	3.308	0.001	0.022	3B	AA_kinase
TraesCS3B02G583000	162.428	0.646	0.208	3.100	0.002	0.036	3B	NA
TraesCS3B02G592500	1126.585	-0.355	0.074	-4.805	0.000	0.000	3B	NA
TraesCS3B02G596400	73.316	-3.591	0.812	-4.422	0.000	0.001	3B	p450
TraesCS3B02G596800	121.646	-2.926	0.906	-3.229	0.001	0.027	3B	p450
TraesCS3B02G597000	143.464	-3.266	0.934	-3.498	0.000	0.014	3B	p450
TraesCS3B02G597900	2903.821	1.471	0.393	3.745	0.000	0.007	3B	NA
TraesCS3B02G612200	4560.536	1.253	0.382	3.279	0.001	0.024	3B	Myb_DNA-binding

Table 3.3. Spring wheat ANOVA results – significant metabolites from the complete dataset

Description	m/z	R.T.	Formula	Stage F val	Stage adj p-val	Allele F val	Allele adj p-val	Interaction F val	Interaction adj p-val
ethyl (S)-3-hydroxybutyrate glucoside	317.12	9.41	C12H22O8	0.07	0.91	3.33	0.04	2.84	0.30
O-glycosyl compound	527.16	0.80	C18H32O16	11.98	0.02	3.37	0.04	1.12	0.81
fucose 1-phosphate	227.03	8.86	C6H13O8P	11.80	0.03	4.44	0.01	1.58	0.72
glycerol 3-phosphate	173.02	7.88	C3H9O6P	3.55	0.21	3.82	0.02	0.38	0.95
cyanidin 3-rutinoside	596.17	6.85	C27H31O15+	11.55	0.03	2.57	0.09	1.67	0.72
4-(4-Hydroxyphenyl)-2-butanone O-[2-galloyl-6-p-coumaroylglucoside]	607.19	0.88	C32H32O13	25.30	0.00	7.79	0.00	3.40	0.19
3-Hydroxy-4-butanolide	287.07	0.82	C10H16O8	1.33	0.51	4.03	0.02	2.74	0.30
1-O-Caffeoyl-(b-D-glucose 6-O-sulfate)	445.04	1.21	C15H18O12S	53.82	0.00	3.17	0.05	1.17	0.81
prunin 6"-O-gallate	569.13	7.98	C28H26O14	10.14	0.03	2.95	0.06	2.30	0.49
sucrose	365.11	2.41	C12H22O11	15.19	0.01	2.47	0.10	1.29	0.81
benzoyl glucuronide (benzoic acid)	321.06	1.42	C13H14O8	0.11	0.88	3.32	0.04	0.53	0.92
feruloyl-2-hydroxyputrescine	303.13	0.85	C14H20N2O4	1.00	0.60	16.51	0.00	3.69	0.19
caffeoylmalic acid	279.05	1.55	C13H12O8	24.39	0.00	1.42	0.34	0.85	0.87
1-Phosphatidyl-1D-myo-inositol 3-phosphate	471.03	0.89	C11H20O16P2	21.39	0.00	3.99	0.02	1.10	0.81
cyclocalamin	485.22	0.83	C27H34O9	4.52	0.17	9.99	0.00	1.61	0.72
cinncassiol D4	375.21	3.12	C20H32O5	0.01	0.97	3.59	0.04	0.64	0.88
8-Hydroxypinoresinol	357.13	6.35	C20H22O7	11.85	0.02	1.60	0.27	0.95	0.84
2,2,6,6-Tetramethyl-4-piperidinone	138.13	0.81	C9H17NO	0.16	0.88	18.27	0.00	1.05	0.81

Table 3.3 continued

niacinamide	123.06	10.51	C6H6N2O	10.33	0.03	2.50	0.10	0.45	0.95
semilicoisoflavone B	335.09	10.57	C20H16O6	3.46	0.21	3.22	0.05	0.43	0.95
methyl 3-(2,3-dihydroxy-3-methylbutyl)-4-hydroxybenzoate	277.11	1.11	C13H18O5	0.18	0.87	3.32	0.04	1.00	0.83
4-hydroxy benzeneacetonitrile	156.04	10.55	C8H7NO	0.59	0.72	3.42	0.04	3.42	0.19
N,N'-Bis(gamma-glutamyl)cystine	499.12	8.08	C16H26N4O10S2	7.10	0.09	3.22	0.05	1.16	0.81
riboflavin	359.14	0.84	C17H20N4O6	3.98	0.19	13.99	0.00	2.83	0.30
(-)-dioxibrassinin	291.02	8.12	C11H12N2O2S2	15.49	0.01	5.91	0.00	2.89	0.30
(+)-12a-hydroxypachyrrhizone	365.06	2.34	C20H14O8	16.55	0.01	2.02	0.17	1.57	0.72
4'-methyl-(-)-epigallocatechin 3'-glucuronide	519.11	8.26	C22H24O13	10.67	0.03	1.72	0.23	1.13	0.81
kievitol	357.13	0.84	C20H22O7	9.12	0.05	13.09	0.00	2.93	0.30
dumetorine	206.15	5.62	C13H21NO2	0.92	0.62	3.85	0.02	1.83	0.70
mevalonic acid-5P	229.05	7.88	C6H13O7P	1.08	0.58	3.44	0.04	1.09	0.81
flavone	303.05	0.85	C15H10O7	0.00	0.98	11.37	0.00	4.08	0.19
3,5-digalloylepicatechin	577.09	1.24	C29H22O14	34.91	0.00	4.70	0.01	1.74	0.70
santene hydrate	123.12	6.30	C9H16O	3.05	0.24	5.25	0.00	0.86	0.87
benzenoid	105.07	6.29	C8H10O	0.24	0.86	3.37	0.04	0.80	0.88
2-O-galloylgalactaric acid	385.04	1.46	C13H14O12	14.72	0.01	1.16	0.45	0.84	0.88

Table 3.4. Spring wheat ANOVA results – significant metabolites from the NIL dataset

Description	m/z	R.T.	Formula	Stage F val	Stage adj p-val	Allele F val	Allele adj p-val	Interaction F val	Interaction adj p-val
benzyl β-primeveroside	403.16	6.48	C18H26O10	0.02	0.94	5.27	0.02	1.82	0.68
fucose 1-phosphate	227.03	8.86	C6H13O8P	11.06	0.04	3.44	0.08	1.18	0.72
riboflavin	359.14	0.84	C17H20N4O6	11.94	0.04	11.83	0.00	2.90	0.57
flavone	303.05	0.85	C15H10O7	0.04	0.93	20.63	0.00	7.26	0.05
feruloyl-2-hydroxyputrescine	303.13	0.85	C14H20N2O4	0.46	0.72	23.86	0.00	6.38	0.06
caffeoylmalic acid	279.05	1.55	C13H12O8	17.30	0.01	1.60	0.32	1.00	0.77
1-phosphatidyl-1D-myo-inositol 3-phosphate	471.03	0.89	C11H20O16P2	10.65	0.04	4.23	0.05	1.22	0.72
2,2,6,6-tetramethyl-4-piperidinone	138.13	0.81	C9H17NO	0.39	0.75	12.99	0.00	1.62	0.68
(-)-dioxibrassinin	291.02	8.12	C11H12N2O2S2	11.22	0.04	9.03	0.00	3.01	0.57
3,5-digalloylepicatechin	577.09	1.24	C29H22O14	22.38	0.00	5.61	0.01	1.55	0.68
benzenoid	105.07	6.29	C8H10O	0.47	0.72	6.66	0.01	1.92	0.68
O-glycosyl compound	527.16	0.80	C18H32O16	12.56	0.04	3.58	0.08	1.01	0.77
4'-methyl-(-)-epigallocatechin 3'-glucuronide	519.11	8.26	C22H24O13	13.91	0.03	2.18	0.17	0.76	0.83
4-(4-hydroxyphenyl)-2-butanone O-[2-galloyl-6-p-coumaroylglucoside]	607.19	0.88	C32H32O13	21.93	0.00	10.31	0.00	4.16	0.30
3-hydroxy-4-butanolide	287.07	0.82	C10H16O8	2.25	0.38	6.95	0.01	4.07	0.30
1-O-caffeoyl-(b-D-glucose 6-O-sulfate)	445.04	1.21	C15H18O12S	46.23	0.00	3.49	0.08	0.36	0.91

Table 3.4 continued

3,5,6-trihydroxy-3',4',7-trimethoxyflavone 3-glucuronide	519.12	6.28	C24H24O14	5.86	0.15	6.10	0.01	1.63	0.68
benzoyl glucuronide (benzoic acid)	321.06	1.42	C13H14O8	0.14	0.87	5.86	0.01	0.96	0.77
cyclocalamin	485.22	0.83	C27H34O9	7.25	0.12	8.10	0.00	1.72	0.68
8-hydroxypinoresinol	357.13	6.35	C20H22O7	12.25	0.04	1.83	0.26	1.36	0.71
6-gingesulfonic acid	341.14	6.30	C17H26O6S	10.91	0.04	3.06	0.11	3.29	0.49
glabrin C	813.49	0.85	C41H64N8O9	0.02	0.94	5.60	0.01	2.52	0.59
4-hydroxy benzeneacetonitrile	156.04	5.12	C8H7NO	4.70	0.21	7.28	0.01	2.74	0.57
kievitol	357.13	0.84	C20H22O7	24.90	0.00	11.53	0.00	2.59	0.57
dumetorine	206.15	5.62	C13H21NO2	0.17	0.85	5.00	0.03	2.20	0.64
castaneiolide	403.17	6.36	C22H28O8	4.99	0.20	4.57	0.05	0.15	0.99
hirsutin	256.08	4.99	C10H19NOS2	1.36	0.54	4.53	0.05	2.33	0.64
santene hydrate	123.12	6.30	C9H16O	5.00	0.20	10.20	0.00	1.29	0.72
2-O-galloylgalactaric acid	385.04	1.46	C13H14O12	12.76	0.04	2.01	0.21	0.55	0.87

Table 3.5. Spring wheat ANOVA results – significant metabolites from the *3BLb*/*3BLc* dataset

Description	**m/z**	**R.T.**	**Formula**	**Allele F val**	**Allele adj p-val**	**Stage F val**	**Stage adj p-val**	**Interaction F val**	**Interaction adj p-val**
(-)-dioxibrassinin	291.02	8.12	C11H12N2O2S2	42.17	0.03	4.28	0.42	9.76	0.43
mevalonic acid-5P	229.05	7.88	C6H13O7P	34.52	0.03	11.24	0.17	5.23	0.64
4-hydroxy benzeneacetonitrile	156.04	5.12	C8H7NO	33.07	0.03	103.94	0.00	1.05	0.94

Table 3.5 continued

3-hydroxy-4-butanolide	287.07	0.82	C10H16O8	26.79	0.03	20.84	0.09	12.17	0.43
flavone	303.05	0.85	C15H10O7	25.22	0.03	21.71	0.09	13.22	0.43
feruloyl-2-hydroxyputrescine	303.13	0.85	C14H20N2O4	24.05	0.03	20.58	0.09	9.58	0.43

Table 3.6. Spring wheat ANOVA results – significant metabolites from the *3BLb*/*3BLa* dataset

Description	**m/z**	**R.T.**	**Formula**	**Allele F val**	**Allele adj p-val**	**Stage F val**	**Stage adj p-val**	**Interaction F val**	**Interaction adj p-val**
S-(hydroxymethyl) glutathione	338.10	6.09	C11H19N3O7S	32.69	0.05	17.89	0.11	17.28	0.14
citronellyl anthranilate	298.18	5.62	C17H25NO2	29.90	0.05	10.95	0.17	19.94	0.12
6-gingesulfonic acid	341.14	6.30	C17H26O6S	28.05	0.05	45.03	0.03	21.70	0.12
3,5,6-trihydroxy-3',4',7-trimethoxy flavone 3-glucuronide	519.12	6.28	C24H24O14	24.37	0.05	0.52	0.75	12.00	0.21

Table 3.7. Transcripts with significant allelic differences in durum wheat

Gene ID	base Mean	log2 Fold Change (LFC)	LFC Std Err	Wald stat	p-val	adj p-val	chr	Gene description	Transcription factor family
TRITD3Av1G000170	1342.121	-1.242	0.304	-4.093	0.000	0.003	3A	Eukaryotic translation initiation factor 3 subunit A	-
TRITD3Av1G000420	19.682	2.656	0.747	3.554	0.000	0.014	3A	Receptor kinase-like protein Xa21	LRRNT_2
TRITD3Av1G001240	3706.951	-1.225	0.285	-4.295	0.000	0.001	3A	Glutamate decarboxylase	-
TRITD3Av1G001460	92.390	1.806	0.577	3.132	0.002	0.039	3A	Protein O-linked-mannose β-1,4-N-acetylglucosaminyl transferase 2	-
TRITD3Av1G001570	14.335	-7.193	1.674	-4.297	0.000	0.001	3A	Oxidoreductase NAD-binding domain	NAD_ binding_1
TRITD3Av1G003800	90.810	9.920	2.200	4.509	0.000	0.001	3A	Domain of unknown function	-
TRITD3Av1G003810	16.870	3.537	0.768	4.608	0.000	0.000	3A	Probable glycosyltransferase	-
TRITD3Av1G003860	47.489	1.420	0.432	3.289	0.001	0.027	3A		-
TRITD3Av1G006580	10.894	-6.770	1.851	-3.658	0.000	0.010	3A	Protein FAR1-RELATED SEQUENCE 5	FAR1
TRITD3Av1G007220	3.000	4.775	1.360	3.511	0.000	0.015	3A	E3 ubiquitin-protein ligase SINAT3	-
TRITD3Av1G008620	112.360	-1.593	0.336	-4.748	0.000	0.000	3A	Deoxyhypusine synthase	-

Table 3.7 continued

TRITD3Av1G013910	105.156	2.095	0.668	3.136	0.002	0.038	3A	Protein ASPARTIC PROTEASE IN GUARD CELL 1	-
TRITD3Av1G014000	119.792	0.760	0.237	3.201	0.001	0.033	3A	Probable LRR receptor-like serine/threonine-protein kinase	-
TRITD3Av1G015030	2780.927	-0.668	0.208	-3.220	0.001	0.032	3A	Protein NRT1/ PTR FAMILY 8.1	-
TRITD3Av1G021050	218.799	3.508	0.704	4.983	0.000	0.000	3A	Histone H2B.3	Histone
TRITD3Av1G029140	29.558	5.664	1.360	4.166	0.000	0.002	3A	Mini-chromosome maintenance complex-binding protein	-
TRITD3Av1G030270	233.009	-3.028	0.563	-5.382	0.000	0.000	3A		-
TRITD3Av1G031000	33.942	-1.191	0.373	-3.192	0.001	0.034	3A	GABA transporter 1	Aa_trans
TRITD3Av1G033650	1408.401	-0.587	0.189	-3.101	0.002	0.041	3A	Clavaminate synthase-like protein	-
TRITD3Av1G035810	404.481	-0.587	0.167	-3.511	0.000	0.015	3A	Protein of unknown function	DUF295
TRITD3Av1G040140	118.234	2.081	0.635	3.276	0.001	0.028	3A		-
TRITD3Av1G042130	7.229	-3.258	0.980	-3.324	0.001	0.025	3A		-
TRITD3Av1G042320	149.618	-1.297	0.405	-3.200	0.001	0.033	3A	ABC transporter C family member 8	ABC_tran
TRITD3Av1G044230	11.821	-6.419	1.725	-3.721	0.000	0.008	3A		-
TRITD3Av1G045640	191.626	0.686	0.180	3.803	0.000	0.007	3A	Transcription factor HY5	bZIP_1

Table 3.7 continued

TRITD3Av1G048540	26.927	-1.909	0.596	-3.204	0.001	0.033	3A	Probable RNA-dependent RNA polymerase 4	-
TRITD3Av1G048550	8.047	-3.126	0.685	-4.567	0.000	0.001	3A	Probable RNA-dependent RNA polymerase 3	-
TRITD3Av1G049020	214.575	2.173	0.603	3.604	0.000	0.012	3A	Chitinase 10	-
TRITD3Av1G058460	1085.563	2.184	0.440	4.969	0.000	0.000	3A	D-3-phosphoglycerate dehydrogenase 2	2-Hacid_dh_C
TRITD3Av1G059230	747.738	-0.766	0.238	-3.217	0.001	0.032	3A	Uncharacterized conserved protein	-
TRITD3Av1G062490	488.793	0.958	0.242	3.958	0.000	0.004	3A	Reticulon-like protein B1	-
TRITD3Av1G078430	147.690	-4.787	0.729	-6.569	0.000	0.000	3A		-
TRITD3Av1G082700	30.394	2.383	0.768	3.104	0.002	0.041	3A	Flowering-promoting factor 1-like protein 1	-
TRITD3Av1G086300	224.537	0.412	0.093	4.442	0.000	0.001	3A	Alpha-1,3/1,6-mannosyltransferase alg-2	Glycos_transf_1
TRITD3Av1G086990	6.731	3.808	1.011	3.766	0.000	0.007	3A		-
TRITD3Av1G090200	17.898	2.697	0.840	3.211	0.001	0.032	3A	Protein MIZU-KUSSEI 1	-
TRITD3Av1G095130	291.880	1.775	0.526	3.375	0.001	0.021	3A		-
TRITD3Av1G095500	97.967	0.920	0.274	3.358	0.001	0.022	3A	Dof zinc finger protein DOF3.3	zf-Dof
TRITD3Av1G106360	78.513	25.724	2.398	10.726	0.000	0.000	3A	Ulp1 protease family, C-terminal catalytic domain	Peptidase_C48

Table 3.7 continued

TRITD3Av1G109260	827.850	-0.513	0.167	-3.077	0.002	0.044	3A	Protein LNK1	-
TRITD3Av1G124040	313.266	-1.075	0.317	-3.395	0.001	0.021	3A		-
TRITD3Av1G144700	366.683	-0.483	0.137	-3.530	0.000	0.014	3A	Uncharacterized protein	-
TRITD3Av1G145810	384.723	-0.559	0.171	-3.260	0.001	0.029	3A	Factor of DNA methylation 2	-
TRITD3Av1G154670	560.642	3.788	1.066	3.552	0.000	0.014	3A	Zinc finger CCCH domain-containing protein 9	zf-CCCH
TRITD3Av1G155240	18.414	3.069	0.980	3.132	0.002	0.039	3A	Auxin efflux carrier component 3a	-
TRITD3Av1G160250	29.380	1.387	0.411	3.378	0.001	0.021	3A	Werner Syndrome-like exonuclease	3_5_exonuc
TRITD3Av1G161840	301.742	3.374	0.717	4.706	0.000	0.000	3A	Mannan endo-1,4-β-mannosidase 1	-
TRITD3Av1G162410	1352.366	0.749	0.218	3.444	0.001	0.018	3A	Mitogen-activated protein kinase 8	Pkinase
TRITD3Av1G165580	201.311	2.358	0.689	3.422	0.001	0.019	3A	Fasciclin-like arabinogalactan protein 11	-
TRITD3Av1G166770	225.062	0.927	0.259	3.585	0.000	0.012	3A	Protein PLASTID MOVEMENT IMPAIRED 1-RELATED 1	-
TRITD3Av1G169780	87.694	7.526	1.598	4.710	0.000	0.000	3A		-
TRITD3Av1G170970	9.421	4.689	1.233	3.804	0.000	0.007	3A	Salicylate carboxymethyltransferase	-
TRITD3Av1G171330	9.325	4.947	1.191	4.152	0.000	0.002	3A	ABC transporter D family member 1	ABC_tran

Table 3.7 continued

TRITD3Av1G172350	150.719	0.851	0.206	4.132	0.000	0.002	3A	ABC transporter B family member 11	ABC_membrane
TRITD3Av1G174220	1271.874	-0.425	0.080	-5.335	0.000	0.000	3A	DNA repair helicase XPB1	-
TRITD3Av1G174790	59.576	4.057	0.692	5.858	0.000	0.000	3A		-
TRITD3Av1G178610	30.035	-2.171	0.676	-3.212	0.001	0.032	3A		-
TRITD3Av1G181730	171.435	1.221	0.293	4.171	0.000	0.002	3A	VQ motif-containing protein 4	-
TRITD3Av1G183900	58.651	1.930	0.422	4.569	0.000	0.001	3A	Polynucleotide 3'-phosphatase ZDP	-
TRITD3Av1G185620	85.797	0.599	0.163	3.667	0.000	0.010	3A		-
TRITD3Av1G185730	107.995	-6.749	1.366	-4.940	0.000	0.000	3A	Senescence regulator	-
TRITD3Av1G185910	77.919	5.451	1.170	4.658	0.000	0.000	3A	MADS-box transcription factor 32	SRF-TF
TRITD3Av1G186560	19.012	4.332	0.686	6.311	0.000	0.000	3A	Hexokinase-9	-
TRITD3Av1G186620	205.536	-0.309	0.095	-3.270	0.001	0.028	3A	Probable acyl-[acyl-carrier-protein]--UDP-N-acetylglucosamine O-acyltransferase, mitochondrial	-
TRITD3Av1G187140	833.048	-0.596	0.169	-3.523	0.000	0.015	3A	Fe-S cluster assembly factor HCF101, chloroplastic	-
TRITD3Av1G187330	12.643	7.215	0.942	7.663	0.000	0.000	3A		-

Table 3.7 continued

TRITD3Av1G188230	392.536	0.570	0.181	3.145	0.002	0.038	3A	Protein of unknown function (DUF1084)	-
TRITD3Av1G188560	77.525	-0.700	0.220	-3.183	0.001	0.034	3A	Probable lipid-A-disaccharide synthase, mitochondrial	-
TRITD3Av1G188730	87.575	2.048	0.218	9.400	0.000	0.000	3A	F-box protein SKIP8	UVR
TRITD3Av1G191050	690.580	0.674	0.189	3.567	0.000	0.013	3A	Phosphatidate cytidylyltransferase 1	-
TRITD3Av1G191120	149.064	1.700	0.453	3.755	0.000	0.008	3A	Domain of unknown function	-
TRITD3Av1G194130	989.560	-0.386	0.121	-3.185	0.001	0.034	3A	BSD domain	-
TRITD3Av1G199000	33.138	1.048	0.328	3.192	0.001	0.034	3A	BTB/POZ and MATH domain-containing protein 1	BTB
TRITD3Av1G199310	100.213	1.318	0.295	4.469	0.000	0.001	3A	Exonuclease 1	XPG_I
TRITD3Av1G200360	791.088	1.735	0.523	3.315	0.001	0.025	3A		-
TRITD3Av1G200810	26.011	1.678	0.550	3.051	0.002	0.047	3A		-
TRITD3Av1G200980	589.780	0.447	0.139	3.221	0.001	0.032	3A	Katanin p80 WD40 repeat-containing subunit B1 homolog	WD40
TRITD3Av1G201160	8.820	-2.452	0.598	-4.099	0.000	0.003	3A	Protein ULTRAPETALA 1	-

Table 3.7 continued

TRITD3Av1G201290	412.204	1.232	0.120	10.227	0.000	0.000	3A	Vacuolar protein sorting-associated protein 28 homolog 1	-
TRITD3Av1G202240	355.476	1.154	0.221	5.226	0.000	0.000	3A	Receptor-like serine/threonine-protein kinase SD1-8	Pkinase_Tyr
TRITD3Av1G204220	23.037	-2.266	0.746	-3.039	0.002	0.048	3A	Domain of unknown function	-
TRITD3Av1G204990	335.129	0.678	0.203	3.333	0.001	0.024	3A	Probable glycerol-3-phosphate dehydrogenase [NAD(+)] 2, cytosolic	NAD_Gly3P_dh_C
TRITD3Av1G206930	317.785	-0.657	0.180	-3.662	0.000	0.010	3A	FAD dependent oxidoreductase	DAO
TRITD3Av1G208780	2947.045	0.731	0.227	3.216	0.001	0.032	3A	NADH--cytochrome b5 reductase 1	FAD_binding_6
TRITD3Av1G214130	1675.620	-0.451	0.128	-3.537	0.000	0.014	3A	UPF0051 protein ABCI8, chloroplastic	-
TRITD3Av1G215260	1063.260	1.299	0.304	4.271	0.000	0.002	3A	Putative respiratory burst oxidase homolog protein H	FAD_binding_8
TRITD3Av1G215550	2263.067	0.398	0.131	3.030	0.002	0.049	3A	Protein GPR107	-
TRITD3Av1G216430	18.540	1.882	0.520	3.618	0.000	0.011	3A	AAA-ATPase	AAA

Table 3.7 continued

TRITD3Av1G217470	43.472	5.520	1.100	5.016	0.000	0.000	3A	Bifunctional purine biosynthesis protein PurH	-
TRITD3Av1G217890	272.748	-1.342	0.325	-4.126	0.000	0.002	3A	Protein of unknown function	-
TRITD3Av1G218000	991.000	0.604	0.191	3.161	0.002	0.036	3A	Protein YIPF6	-
TRITD3Av1G220090	3.199	3.595	1.187	3.028	0.002	0.049	3A	Hevamine-A	-
TRITD3Av1G221410	18.485	1.084	0.309	3.513	0.000	0.015	3A	F-box/LRR-repeat protein 13	-
TRITD3Av1G223350	172.778	-1.161	0.246	-4.720	0.000	0.000	3A	Probable anion transporter 3, chloroplastic	MFS_1
TRITD3Av1G224680	693.859	-0.407	0.112	-3.637	0.000	0.011	3A	ATP-dependent zinc metalloprotease FTSH 8, mitochondrial	AAA
TRITD3Av1G224750	65.849	4.426	1.226	3.611	0.000	0.012	3A	Putative laccase-5	-
TRITD3Av1G227030	41.870	-3.809	0.818	-4.659	0.000	0.000	3A	Magnesium transporter MRS2-E >A2WXD3.1	-
TRITD3Av1G230320	147.932	-25.673	2.496	-10.285	0.000	0.000	3A		-
TRITD3Av1G230480	76.928	1.467	0.397	3.697	0.000	0.009	3A		-
TRITD3Av1G231760	101.701	1.294	0.419	3.090	0.002	0.043	3A	GDP-L-galactose phosphorylase 1	-
TRITD3Av1G232670	257.400	1.399	0.345	4.054	0.000	0.003	3A	Formin-like protein 1	FH2
TRITD3Av1G235540	947.901	2.572	0.559	4.601	0.000	0.000	3A	Amino acid permease 3	Aa_trans

Table 3.7 continued

TRITD3Av1G237720	132.373	0.524	0.166	3.146	0.002	0.037	3A		-
TRITD3Av1G240330	180.838	9.388	2.204	4.260	0.000	0.002	3A	Probable lipid transfer	-
TRITD3Av1G240760	1121.568	0.447	0.117	3.816	0.000	0.006	3A	Protein YLS7	-
TRITD3Av1G241970	31.936	2.118	0.578	3.668	0.000	0.010	3A		-
TRITD3Av1G242650	831.462	1.575	0.352	4.473	0.000	0.001	3A	Small ubiquitin-related modifier 1	-
TRITD3Av1G243570	24.791	8.142	1.841	4.422	0.000	0.001	3A	Probable histone acetyltransferase HAC-like 1	-
TRITD3Av1G245100	16.515	4.741	1.423	3.331	0.001	0.024	3A	Uncharacterized protein	-
TRITD3Av1G246370	457.449	2.354	0.557	4.228	0.000	0.002	3A	Probable histidine kinase 3	HATPase_c
TRITD3Av1G248570	8.753	4.466	1.251	3.569	0.000	0.013	3A	Auxin responsive protein	Auxin_ inducible
TRITD3Av1G248960	84.465	0.542	0.167	3.247	0.001	0.030	3A		-
TRITD3Av1G248980	149.250	1.588	0.309	5.138	0.000	0.000	3A	Probable protein S-acyltransferase 7	-
TRITD3Av1G250420	289.112	0.268	0.088	3.032	0.002	0.049	3A	Glutaminyl-peptide cyclotransferase	-
TRITD3Av1G255060	362.258	2.066	0.589	3.509	0.000	0.015	3A	Zinc finger protein MAGPIE	-
TRITD3Av1G256040	1824.045	-1.413	0.417	-3.390	0.001	0.021	3A	Spermatogenesis-associated protein 20	-
TRITD3Av1G256050	116.910	-2.132	0.420	-5.070	0.000	0.000	3A	Cytochrome c1 2, heme protein, mitochondrial	-

Table 3.7 continued

TRITD3Av1G260200	25.088	7.399	1.418	5.217	0.000	0.000	3A	Hydroxyethyl thiazole kinase	-
TRITD3Av1G264270	9.170	-21.754	2.974	-7.315	0.000	0.000	3A	Glucan endo-1,3- β-glucosidase, acidic isoform	Glyco_ hydro_17
TRITD3Av1G266030	4.593	5.688	1.859	3.060	0.002	0.046	3A	No apical meristem (NAM) protein	NAM
TRITD3Av1G267280	74.462	4.808	0.917	5.245	0.000	0.000	3A	Protein of unknown function (DUF1668)	-
TRITD3Av1G268100	148.508	-1.381	0.443	-3.119	0.002	0.040	3A	CSC1-like protein At3g54510	-
TRITD3Av1G268570	3.325	4.316	1.261	3.423	0.001	0.019	3A	Arabidopsis protein of unknown function	-
TRITD3Av1G269120	5.394	4.989	1.515	3.293	0.001	0.027	3A	Serine/threonine protein kinase OSK4	Pkinase
TRITD3Av1G269130	10.938	7.023	1.843	3.812	0.000	0.007	3A		-
TRITD3Av1G269650	229.874	0.519	0.160	3.255	0.001	0.029	3A	Probable ADP-ribosylation factor GTPase-activating protein AGD11	C2
TRITD3Av1G269690	175.493	-0.634	0.182	-3.493	0.000	0.016	3A	Lycopene cyclase protein	-
TRITD3Av1G269870	641.519	-0.352	0.101	-3.500	0.000	0.015	3A	β-galactosidase	-
TRITD3Av1G270710	49.021	8.279	2.121	3.904	0.000	0.005	3A	GPI-anchored protein LLG1	-

Table 3.7 continued

TRITD3Av1G271230	113.046	1.349	0.282	4.792	0.000	0.000	3A	Cysteine-rich receptor-like protein kinase 6	Pkinase_Tyr
TRITD3Av1G271240	36.050	5.029	0.994	5.058	0.000	0.000	3A	Cysteine-rich receptor-like protein kinase 25	Pkinase_Tyr
TRITD3Av1G273770	100.636	0.868	0.239	3.634	0.000	0.011	3A	Probable RNA-binding protein ARP1	RRM_1
TRITD3Av1G277450	28.539	-6.008	1.167	-5.146	0.000	0.000	3A	ABA/WDS induced protein	-
TRITD3Av1G277830	81.521	0.925	0.271	3.414	0.001	0.020	3A	VAMP-like protein YKT61	-
TRITD3Av1G278230	285.994	-0.937	0.194	-4.840	0.000	0.000	3A	KH domain-containing protein	KH_1
TRITD3Av1G278240	436.228	-1.913	0.391	-4.898	0.000	0.000	3A		-
TRITD3Av1G278270	24.795	-7.925	1.832	-4.325	0.000	0.001	3A		-
TRITD3Av1G278300	15.718	-7.316	1.854	-3.947	0.000	0.004	3A		-
TRITD3Av1G279510	22.849	-2.026	0.480	-4.217	0.000	0.002	3A	Diphospho mevalonate decarboxylase MVD2	GHMP_ kinases
TRITD3Av1G280920	602.647	-0.829	0.195	-4.242	0.000	0.002	3A	Eukaryotic translation initiation factor 4E-1	-
TRITD3Av1G281830	10256.454	0.286	0.091	3.139	0.002	0.038	3A	Cytochrome b5	-
TRITD3Av1G283400	223.498	1.081	0.339	3.190	0.001	0.034	3A		-
TRITD3Av1G286070	406.095	1.594	0.377	4.230	0.000	0.002	3A	Soluble inorganic pyrophosphatase 4	Pyro phosphatase
TRITD3Av1G287170	6.055	-5.444	1.602	-3.399	0.001	0.020	3A		-

Table 3.7 continued

TRITD3Av1G287390	108.662	-10.092	1.867	-5.406	0.000	0.000	3A	DnaJ protein P58IPK homolog	DnaJ
TRITD3Av1G287400	149.997	-9.390	1.854	-5.066	0.000	0.000	3A	Zinc finger CCCH domain-containing protein 47	-

Table 3.8. Transcripts with significant growth stage differences in durum wheat

Gene ID	**base Mean**	**log2 Fold Change (LFC)**	**LFC Std Error**	**Wald Stat**	**p-val**	**adj p-val**	**chr**	**Gene description**	**Transcription factor family**
TRITD3Av1G000830	254.880	5.299	1.093	4.850	0.000	0.000	3A	Spermidine hydroxycinnamoyl transferase 2	-
TRITD3Av1G000870	215.347	-1.008	0.325	-3.098	0.002	0.033	3A	CSC1-like protein ERD4	-
TRITD3Av1G001140	6.201	-5.140	1.195	-4.303	0.000	0.002	3A	Protein of unknown function	-
TRITD3Av1G001300	171.093	-1.085	0.348	-3.121	0.002	0.031	3A	Protein of unknown function	-
TRITD3Av1G001470	577.507	-0.498	0.151	-3.293	0.001	0.022	3A	Leucine--tRNA ligase, chloroplastic/mitochondrial	-
TRITD3Av1G001500	349.332	0.822	0.253	3.246	0.001	0.024	3A	Alanyl-tRNA editing protein AlaX-L	-
TRITD3Av1G002020	73.959	-4.699	1.149	-4.089	0.000	0.003	3A		-
TRITD3Av1G002220	663.547	-0.528	0.174	-3.036	0.002	0.037	3A	Tetratricopeptide repeat	-

Table 3.8 continued

TRITD3Av1G007160	115.198	-1.505	0.511	-2.943	0.003	0.044	3A		-
TRITD3Av1G007370	389.559	-1.346	0.333	-4.046	0.000	0.003	3A	Lecithin-cholesterol acyltransferase-like 1	-
TRITD3Av1G007860	15.178	-2.028	0.657	-3.087	0.002	0.034	3A		-
TRITD3Av1G008330	102.188	-1.120	0.283	-3.951	0.000	0.004	3A	DUF761-associated sequence motif	-
TRITD3Av1G009370	99.097	2.200	0.740	2.972	0.003	0.042	3A	PF14543:Xylanase inhibitor N-terminal|PF14541:Xylanase inhibitor C-terminal	-
TRITD3Av1G010870	14.556	-3.620	1.117	-3.242	0.001	0.025	3A	Mannose-6-phosphate isomerase 1	-
TRITD3Av1G013230	20295.891	-1.014	0.285	-3.554	0.000	0.012	3A	Photosystem I reaction center subunit XI, chloroplastic	-
TRITD3Av1G013810	50.533	-1.276	0.282	-4.529	0.000	0.001	3A	Calcineurin B-like protein 9	-
TRITD3Av1G014600	376.476	-0.523	0.128	-4.094	0.000	0.003	3A	Protein root UVB sensitive 5	-
TRITD3Av1G020840	155.795	0.996	0.260	3.833	0.000	0.006	3A	Domain of unknown function	-
TRITD3Av1G021320	335.045	-0.508	0.163	-3.122	0.002	0.031	3A		-
TRITD3Av1G023500	1410.087	0.355	0.114	3.106	0.002	0.032	3A	Translocon-associated protein β (TRAPB)	-

Table 3.8 continued

TRITD3Av1G024620	605.655	0.340	0.094	3.607	0.000	0.011	3A	Protein of unknown function	-
TRITD3Av1G027010	185.825	-2.227	0.688	-3.239	0.001	0.025	3A	Galactinol--sucrose galactosyltransferase	-
TRITD3Av1G027590	30.377	-4.033	1.359	-2.968	0.003	0.042	3A	Subtilisin-chymotrypsin inhibitor-2A	-
TRITD3Av1G029290	1179.878	-1.498	0.499	-2.999	0.003	0.039	3A	Probable staphylococcal-like nuclease CAN1	-
TRITD3Av1G031430	174.625	1.818	0.595	3.053	0.002	0.036	3A	Protein DOG1-like 2	-
TRITD3Av1G031600	1040.265	1.064	0.247	4.312	0.000	0.002	3A		-
TRITD3Av1G032470	279.769	-1.640	0.352	-4.662	0.000	0.000	3A		-
TRITD3Av1G033610	418.747	-1.531	0.359	-4.264	0.000	0.002	3A	Auxin-responsive protein IAA16	-
TRITD3Av1G033650	1408.401	0.668	0.189	3.531	0.000	0.013	3A	Clavaminate synthase-like protein	-
TRITD3Av1G035590	393.548	-2.618	0.655	-3.997	0.000	0.004	3A	17.5 kDa class II heat shock protein	-
TRITD3Av1G035960	321.439	-0.751	0.214	-3.517	0.000	0.013	3A		-
TRITD3Av1G039020	411.426	-0.605	0.177	-3.423	0.001	0.016	3A	Protein LOW PSII ACCUMULATION 1, chloroplastic	-
TRITD3Av1G040140	118.234	-1.905	0.635	-2.998	0.003	0.039	3A		-
TRITD3Av1G040350	104.909	-0.786	0.221	-3.556	0.000	0.012	3A		-

Table 3.8 continued

TRITD3Av1G041150	94.355	0.634	0.201	3.160	0.002	0.029	3A	Plasma membrane glucose-responsive regulator	-
TRITD3Av1G043240	528.563	0.452	0.115	3.927	0.000	0.005	3A	Prefoldin subunit 6	-
TRITD3Av1G043690	109.171	1.912	0.494	3.870	0.000	0.006	3A	UDP-glycosyltransferase 73D1	-
TRITD3Av1G046700	31.717	1.092	0.303	3.598	0.000	0.011	3A		-
TRITD3Av1G047670	1116.276	-2.189	0.671	-3.263	0.001	0.023	3A		-
TRITD3Av1G048480	624.361	-0.674	0.210	-3.209	0.001	0.026	3A	Probable RNA-dependent RNA polymerase 3	-
TRITD3Av1G049320	1044.853	0.812	0.263	3.088	0.002	0.034	3A	β-glucuronosyltransferase GlcAT14A	-
TRITD3Av1G055030	786.024	-0.529	0.168	-3.149	0.002	0.030	3A	GDSL esterase/lipase At5g45920	-
TRITD3Av1G057860	658.190	-1.852	0.391	-4.737	0.000	0.000	3A	Plant protein 1589 of unknown function (A_thal_3526)	-
TRITD3Av1G058380	1348.080	1.877	0.333	5.638	0.000	0.000	3A		-
TRITD3Av1G059210	288.732	-0.910	0.236	-3.860	0.000	0.006	3A	GDT1-like protein 1, chloroplastic	-
TRITD3Av1G059540	14202.944	-0.934	0.284	-3.285	0.001	0.022	3A	Nodulin-related protein 1	-
TRITD3Av1G061830	607.641	-3.593	1.107	-3.245	0.001	0.024	3A	Late embryogenesis abundant protein Lea14-A	-

Table 3.8 continued

TRITD3Av1G064030	470.433	-0.981	0.329	-2.979	0.003	0.041	3A	Pentatricopeptide repeat-containing protein At5g25630	-
TRITD3Av1G070430	12905.162	-0.752	0.224	-3.353	0.001	0.019	3A	Triose phosphate/phosphate translocator, chloroplastic	-
TRITD3Av1G086690	80.281	-5.515	1.422	-3.878	0.000	0.005	3A	Peroxidase 72	-
TRITD3Av1G087230	272.493	0.281	0.084	3.353	0.001	0.019	3A	Breast carcinoma amplified sequence 2 (BCAS2)	-
TRITD3Av1G090010	1151.012	0.371	0.122	3.041	0.002	0.037	3A	Calmodulin-related protein	-
TRITD3Av1G091010	991.220	1.222	0.291	4.204	0.000	0.002	3A	Peroxidase 24	-
TRITD3Av1G091260	602.953	0.361	0.124	2.905	0.004	0.048	3A	Tim17/Tim22/Tim23/ Pmp24 family	-
TRITD3Av1G091270	1021.717	0.412	0.138	2.993	0.003	0.040	3A	Glutamate--tRNA ligase, cytoplasmic	-
TRITD3Av1G094680	81.289	-1.555	0.429	-3.627	0.000	0.010	3A	E3 ubiquitin-protein ligase RING1	-
TRITD3Av1G102330	2485.344	0.617	0.207	2.977	0.003	0.041	3A	Coatomer subunit β-2	-
TRITD3Av1G115810	19.451	-0.870	0.297	-2.933	0.003	0.045	3A	Protein translocase subunit SECA1, chloroplastic	-
TRITD3Av1G121630	92.916	-1.265	0.328	-3.861	0.000	0.006	3A		-
TRITD3Av1G126920	55.538	-0.946	0.288	-3.285	0.001	0.022	3A	Vesicle transport protein GOT1	-

Table 3.8 continued

TRITD3Av1G127210	507.457	-0.347	0.119	-2.925	0.003	0.046	3A	Zinc protease PQQL-like	-
TRITD3Av1G130160	511.574	-0.699	0.224	-3.116	0.002	0.032	3A	Protein indeterminate-domain 11	-
TRITD3Av1G130900	599.205	1.302	0.273	4.767	0.000	0.000	3A	Domain of unknown function (DUF3511)	-
TRITD3Av1G131180	790.958	0.235	0.063	3.735	0.000	0.008	3A	Vesicle transport v-SNARE 13	-
TRITD3Av1G131550	326.687	0.642	0.206	3.113	0.002	0.032	3A	Protein transport protein Sec61 subunit β	-
TRITD3Av1G134280	808.996	-1.257	0.359	-3.501	0.000	0.014	3A	Protein of unknown function (DUF1118)	-
TRITD3Av1G148540	898.426	-0.769	0.133	-5.774	0.000	0.000	3A	Probable zinc-ribbon domain	-
TRITD3Av1G151270	259.636	1.355	0.304	4.454	0.000	0.001	3A	Uroporphyrinogen-III C-methyltransferase	-
TRITD3Av1G155400	6.819	-3.424	1.064	-3.218	0.001	0.026	3A	Protein MIZU-KUSSEI 1	-
TRITD3Av1G157510	37.364	-4.921	1.245	-3.952	0.000	0.004	3A		-
TRITD3Av1G159730	16.737	-4.225	1.223	-3.453	0.001	0.015	3A	Acidic endochitinase	-
TRITD3Av1G160910	8.072	-2.919	0.922	-3.167	0.002	0.029	3A		-
TRITD3Av1G161840	301.742	-2.833	0.717	-3.952	0.000	0.004	3A	Mannan endo-1,4-β-mannosidase 1	-

Table 3.8 continued

TRITD3Av1G163170	109.959	-1.946	0.611	-3.182	0.001	0.028	3A	Protein IQ-DOMAIN 31	-
TRITD3Av1G163370	158.382	-1.568	0.498	-3.149	0.002	0.030	3A	Tryptophan aminotransferase-related protein 4	-
TRITD3Av1G166320	681.481	-0.651	0.210	-3.107	0.002	0.032	3A	Alpha-amylase 3, chloroplastic	-
TRITD3Av1G166580	142.514	-0.806	0.270	-2.988	0.003	0.040	3A		-
TRITD3Av1G168190	414.532	-0.619	0.153	-4.052	0.000	0.003	3A	5'-nucleotidase SurE	-
TRITD3Av1G169980	2604.970	1.123	0.296	3.793	0.000	0.007	3A		-
TRITD3Av1G171720	6380.337	-0.989	0.261	-3.792	0.000	0.007	3A	Protein plastid transcriptionally active 16	-
TRITD3Av1G171990	1888.157	0.382	0.128	2.985	0.003	0.040	3A		-
TRITD3Av1G175780	75.211	-3.072	0.641	-4.795	0.000	0.000	3A	Domain of unknown function	-
TRITD3Av1G175830	250.635	-1.727	0.472	-3.662	0.000	0.009	3A	Domain of unknown function	-
TRITD3Av1G177250	864.792	0.299	0.094	3.170	0.002	0.028	3A	Protein SDE2 homolog	-
TRITD3Av1G179910	1090.296	-0.735	0.191	-3.843	0.000	0.006	3A	Pentatricopeptide repeat-containing protein At1g30610, chloroplastic	-
TRITD3Av1G181860	70.846	-6.587	1.616	-4.076	0.000	0.003	3A	Mannan endo-1,4-β-mannosidase 2	-
TRITD3Av1G183030	32.136	-3.921	0.737	-5.324	0.000	0.000	3A	Auxin-responsive protein IAA6	-

Table 3.8 continued

TRITD3Av1G183610	160.571	0.736	0.243	3.034	0.002	0.037	3A	Serine/threonine-protein phosphatase 7 long form homolog	-
TRITD3Av1G183880	563.985	0.371	0.121	3.081	0.002	0.034	3A	Protein farnesyltransferase subunit β	-
TRITD3Av1G185100	131.591	0.640	0.169	3.797	0.000	0.007	3A	Putative F-box/LRR-repeat protein At5g54820	-
TRITD3Av1G185730	107.995	-4.492	1.363	-3.296	0.001	0.022	3A	Senescence regulator	-
TRITD3Av1G186550	824.705	0.997	0.255	3.917	0.000	0.005	3A	Senescence-associated protein	-
TRITD3Av1G187450	30.453	-2.582	0.597	-4.323	0.000	0.002	3A	zinc-finger of the FCS-type, C2-C2	-
TRITD3Av1G190740	163.221	-0.529	0.166	-3.189	0.001	0.027	3A	Bifunctional riboflavin kinase/FMN phosphatase	-
TRITD3Av1G192090	2262.729	-0.571	0.197	-2.906	0.004	0.048	3A	CAAD domains of cyanobacterial aminoacyl-tRNA synthetase	-
TRITD3Av1G192660	284.239	0.940	0.247	3.806	0.000	0.006	3A	Triacylglycerol lipase SDP1	-
TRITD3Av1G194410	460.552	-1.254	0.220	-5.688	0.000	0.000	3A	Probable xyloglucan glycosyltransferase 1	-

Table 3.8 continued

TRITD3Av1G197960	12908.753	-0.947	0.324	-2.923	0.003	0.046	3A	Photosystem II reaction center W protein, chloroplastic	-
TRITD3Av1G198050	710.744	-0.717	0.225	-3.185	0.001	0.028	3A	Plus-3 domain	-
TRITD3Av1G198480	754.969	1.585	0.423	3.747	0.000	0.007	3A	Jacalin-related lectin 3	-
TRITD3Av1G202460	26.475	-2.119	0.646	-3.281	0.001	0.023	3A		-
TRITD3Av1G204170	696.335	-0.560	0.188	-2.979	0.003	0.041	3A	Protein CHLOROPLAST ENHANCING STRESS TOLERANCE, chloroplastic	-
TRITD3Av1G204520	72.009	-0.635	0.208	-3.054	0.002	0.036	3A	Protein MICRORCHIDIA 6	-
TRITD3Av1G206280	556.124	0.488	0.159	3.077	0.002	0.034	3A	Las1-like	-
TRITD3Av1G213280	987.407	0.832	0.267	3.122	0.002	0.031	3A		-
TRITD3Av1G214380	96.225	-1.712	0.565	-3.031	0.002	0.037	3A	Protein ESKIMO 1	-
TRITD3Av1G214950	483.275	0.310	0.103	3.001	0.003	0.039	3A	Nuclear transcription factor Y subunit B-2	-
TRITD3Av1G215620	1217.181	0.337	0.112	3.010	0.003	0.039	3A	Probable signal peptidase complex subunit 1	-
TRITD3Av1G221080	20.982	-2.454	0.630	-3.899	0.000	0.005	3A		-
TRITD3Av1G224750	65.849	-4.105	1.226	-3.349	0.001	0.019	3A	Putative laccase-5	-
TRITD3Av1G226400	291.793	1.703	0.488	3.488	0.000	0.014	3A		-

Table 3.8 continued

TRITD3Av1G227280	11979.307	-0.972	0.304	-3.199	0.001	0.027	3A	Photosystem II 22 kDa protein, chloroplastic	-
TRITD3Av1G229470	24.958	-3.046	0.892	-3.416	0.001	0.017	3A	β-galactosidase 3	-
TRITD3Av1G230500	635.692	-1.495	0.438	-3.411	0.001	0.017	3A	Uncharacterized protein slp1	-
TRITD3Av1G230630	70.722	-1.352	0.421	-3.213	0.001	0.026	3A	Pentatricopeptide repeat-containing protein	-
TRITD3Av1G233880	890.549	0.334	0.095	3.531	0.000	0.013	3A	Signal recognition particle subunit SRP72	-
TRITD3Av1G234660	98.847	-1.836	0.616	-2.982	0.003	0.041	3A		-
TRITD3Av1G235580	475.998	-1.417	0.447	-3.166	0.002	0.029	3A	NAD(P)H-quinone oxidoreductase subunit N, chloroplastic	-
TRITD3Av1G239010	19.314	-2.468	0.775	-3.184	0.001	0.028	3A	Plant protein of unknown function	-
TRITD3Av1G239120	60734.311	1.449	0.281	5.158	0.000	0.000	3A		-
TRITD3Av1G239280	2770.334	0.399	0.120	3.331	0.001	0.020	3A	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	-
TRITD3Av1G239760	1228.279	-0.837	0.235	-3.559	0.000	0.012	3A	Thioredoxin F, chloroplastic	-
TRITD3Av1G240330	180.838	-8.235	2.203	-3.739	0.000	0.008	3A	Probable lipid transfer	-

Table 3.8 continued

TRITD3Av1G243490	570.682	0.342	0.098	3.483	0.000	0.014	3A	:Zinc finger, C3HC4 type (RING finger)	-
TRITD3Av1G246220	2344.518	-0.783	0.252	-3.104	0.002	0.032	3A	Rhodanese-like domain-containing protein 4, chloroplastic	-
TRITD3Av1G247480	260.473	-0.932	0.303	-3.071	0.002	0.035	3A	Stress enhanced protein 1, chloroplastic	-
TRITD3Av1G248890	10.739	-3.300	1.050	-3.143	0.002	0.030	3A		-
TRITD3Av1G252590	66.666	-1.192	0.317	-3.762	0.000	0.007	3A	Thiamine pyrophosphokinase 1	-
TRITD3Av1G256800	106.345	-1.723	0.567	-3.040	0.002	0.037	3A		-
TRITD3Av1G263940	482.885	0.449	0.156	2.886	0.004	0.049	3A		-
TRITD3Av1G265820	562.604	-1.723	0.525	-3.281	0.001	0.023	3A	Annexin D5	-
TRITD3Av1G271610	183.019	1.342	0.245	5.481	0.000	0.000	3A		-
TRITD3Av1G274660	294.579	-0.949	0.320	-2.968	0.003	0.042	3A	NAD(P)H-quinone oxidoreductase subunit O, chloroplastic	-
TRITD3Av1G276060	907.651	-1.011	0.313	-3.227	0.001	0.025	3A	Chlorophyllide a oxygenase, chloroplastic	-
TRITD3Av1G277380	617.883	-1.983	0.501	-3.961	0.000	0.004	3A	ABA/WDS induced protein	-
TRITD3Av1G279500	1220.388	0.228	0.078	2.915	0.004	0.047	3A		-
TRITD3Av1G282450	25.601	3.324	0.888	3.745	0.000	0.008	3A	Glutelin type-A 2	-

Table 3.8 continued

TRITD3Av1G285900	1007.918	-1.928	0.476	-4.055	0.000	0.003	3A	Pectin acetylesterase 5	-
TRITD3Av1G058460	1085.563	-1.700	0.440	-3.869	0.000	0.006	3A	D-3-phosphoglycerate dehydrogenase 2, chloroplastic	2-Hacid_dh_C
TRITD3Av1G053480	71.493	3.380	1.044	3.238	0.001	0.025	3A	Gibberellin 2-β-dioxygenase 1	2OG-FeII_Oxy
TRITD3Av1G190000	266.669	3.025	0.529	5.715	0.000	0.000	3A	Gibberellin 2-β-dioxygenase	2OG-FeII_Oxy
TRITD3Av1G017880	16.089	4.159	1.234	3.372	0.001	0.018	3A	Amino acid permease 3	Aa_trans
TRITD3Av1G031000	33.942	1.183	0.373	3.170	0.002	0.028	3A	GABA transporter 1	Aa_trans
TRITD3Av1G148510	29.980	-1.265	0.429	-2.949	0.003	0.044	3A	Probable GABA transporter 2	Aa_trans
TRITD3Av1G147380	651.392	-0.621	0.143	-4.350	0.000	0.001	3A	ATP-dependent zinc metalloprotease FTSH 9, chloroplastic/mitochondrial	AAA
TRITD3Av1G216610	87.208	-1.251	0.418	-2.990	0.003	0.040	3A	AAA-ATPase At3g28540	AAA
TRITD3Av1G225440	234.335	0.901	0.300	3.004	0.003	0.039	3A	ABC transporter C family member 3	ABC_tran
TRITD3Av1G041800	279.009	-1.873	0.598	-3.130	0.002	0.031	3A	ABC transporter G family member 31	ABC2_membrane
TRITD3Av1G169970	282.658	1.443	0.496	2.906	0.004	0.048	3A	ABC transporter G family member 6	ABC2_membrane

Table 3.8 continued

TRITD3Av1G186090	616.224	-2.341	0.415	-5.646	0.000	0.000	3A	ABC transporter G family member 38	ABC2_membrane
TRITD3Av1G262760	26.358	-4.810	1.373	-3.502	0.000	0.014	3A	Pimeloyl-[acyl-carrier protein] methyl ester esterase	Abhydrolase_1
TRITD3Av1G089530	920.395	-0.363	0.121	-3.013	0.003	0.038	3A	Putative aconitate hydratase, cytoplasmic	Aconitase
TRITD3Av1G021770	126.689	-1.030	0.340	-3.024	0.002	0.038	3A	Short-chain dehydrogenase TIC 32, chloroplastic	adh_short
TRITD3Av1G063240	702.289	-0.625	0.182	-3.437	0.001	0.016	3A	Probable chlorophyll(ide) b reductase NYC1, chloroplastic	adh_short
TRITD3Av1G239340	3923.157	1.140	0.278	4.101	0.000	0.003	3A	Aldehyde dehydrogenase family 2 member C4	Aldedh
TRITD3Av1G213370	1325.377	-0.618	0.187	-3.310	0.001	0.021	3A	Glutamyl-tRNA(Gln) amidotransferase subunit A	Amidase
TRITD3Av1G260740	252.186	-1.666	0.562	-2.965	0.003	0.042	3A	4-coumarate--CoA ligase-like 7	AMP-binding
TRITD3Av1G203860	207.745	-3.094	0.800	-3.868	0.000	0.006	3A	Ethylene-responsive transcription factor 4	AP2

Table 3.8 continued

TRITD3Av1G267180	167.804	-0.832	0.274	-3.034	0.002	0.037	3A	BTB/POZ domain and ankyrin repeat-containing protein NPR5	BTB
TRITD3Av1G045640	191.626	-0.606	0.180	-3.360	0.001	0.019	3A	Transcription factor HY5	bZIP_1
TRITD3Av1G076970	914.140	-0.760	0.170	-4.484	0.000	0.001	3A	C2 domain-containing protein At1g53590	C2
TRITD3Av1G215220	60.802	1.783	0.496	3.596	0.000	0.011	3A	Zinc finger protein CONSTANS-LIKE 2	CCT
TRITD3Av1G111000	900.880	-0.799	0.190	-4.201	0.000	0.002	3A	Fimbrin-2	CH
TRITD3Av1G036930	149.474	-1.469	0.261	-5.635	0.000	0.000	3A	Probable choline kinase 2	Choline_kinase
TRITD3Av1G170950	1225.266	-0.414	0.134	-3.078	0.002	0.034	3A	Phosphatidylinositol/phosphatidylcholine transfer protein SFH6	CRAL_TRIO_N
TRITD3Av1G053900	542.033	-1.358	0.315	-4.308	0.000	0.002	3A	Plastocyanin-like domain	Cu_bind_like
TRITD3Av1G229570	601.973	-1.183	0.378	-3.132	0.002	0.031	3A	Chaperone protein DnaJ	DnaJ_C
TRITD3Av1G226410	30.696	-1.469	0.372	-3.947	0.000	0.004	3A	Rop guanine nucleotide exchange factor 7	DUF315
TRITD3Av1G204800	87.508	-0.798	0.258	-3.096	0.002	0.033	3A	DNA-(apurinic or apyrimidinic site) lyase, chloroplastic	Exo_endo_phos

Table 3.8 continued

TRITD3Av1G230520	842.765	-1.624	0.540	-3.008	0.003	0.039	3A	Putative F-box protein	F-box
TRITD3Av1G251030	23.989	0.881	0.276	3.192	0.001	0.027	3A	F-box domain	F-box
TRITD3Av1G193640	382.999	0.774	0.258	3.003	0.003	0.039	3A	Formamidase	FmdA_AmdA
TRITD3Av1G083830	198.340	-1.466	0.296	-4.954	0.000	0.000	3A	Uncharacterized oxidoreductase At4g09670	GFO_IDH_MocA
TRITD3Av1G166870	11.158	-2.103	0.714	-2.947	0.003	0.044	3A	β-glucosidase 7	Glyco_hydro_1
TRITD3Av1G264270	9.170	-22.737	2.974	-7.645	0.000	0.000	3A	Glucan endo-1,3- β-glucosidase, acidic isoform	Glyco_hydro_17
TRITD3Av1G273830	7.021	-5.684	1.433	-3.967	0.000	0.004	3A	Glucan endo-1,3- β-glucosidase 8	Glyco_hydro_17
TRITD3Av1G236520	893.869	-0.887	0.216	-4.113	0.000	0.003	3A	UDP-glucuronate:xylan alpha-glucuronosyltransferase 1	Glyco_transf_8
TRITD3Av1G045760	21.854	-2.509	0.771	-3.253	0.001	0.024	3A	Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily	Glyoxalase
TRITD3Av1G007480	186.990	-1.574	0.542	-2.902	0.004	0.048	3A	Transcription factor bHLH35	HLH
TRITD3Av1G231620	738.436	0.819	0.231	3.548	0.000	0.012	3A	Transcription factor bHLH128	HLH

Table 3.8 continued

TRITD3Av1G011720	254.207	2.352	0.787	2.989	0.003	0.040	3A	Heavy metal-associated isoprenylated plant protein 37	HMA
TRITD3Av1G213220	387.060	1.251	0.344	3.641	0.000	0.010	3A	Heavy metal-associated isoprenylated plant protein 45	HMA
TRITD3Av1G253330	1520.472	0.861	0.212	4.057	0.000	0.003	3A	Heavy metal-associated isoprenylated plant protein 36	HMA
TRITD3Av1G166060	1247.005	-0.374	0.124	-3.028	0.002	0.037	3A	Persulfide dioxygenase ETHE1 homolog, mitochondrial	Lactamase_B
TRITD3Av1G086290	71.904	-1.652	0.454	-3.637	0.000	0.010	3A	TLC domain	LAG1
TRITD3Av1G147390	18.122	-2.510	0.761	-3.300	0.001	0.022	3A	Lipase (class 3)	Lipase_3
TRITD3Av1G167910	315.517	-0.948	0.291	-3.257	0.001	0.024	3A	Phospholipase A1-II 5	Lipase_3
TRITD3Av1G055470	64.796	-2.334	0.644	-3.622	0.000	0.010	3A	GDSL esterase/lipase At5g45910	Lipase_GDSL
TRITD3Av1G186350	2620.337	0.959	0.186	5.147	0.000	0.000	3A	NADP-dependent malic enzyme	Malic_M
TRITD3Av1G089410	74.730	3.095	0.690	4.484	0.000	0.001	3A	Probable peptide/nitrate transporter At3g43790	MFS_1

Table 3.8 continued

TRITD3Av1G097240	1484.031	-0.685	0.181	-3.780	0.000	0.007	3A	Probable anion transporter 1, chloroplastic	MFS_1
TRITD3Av1G069870	2635.908	-0.678	0.198	-3.415	0.001	0.017	3A	Probable transcription factor GLK2	Myb_DNA-binding
TRITD3Av1G121270	174.002	-2.157	0.560	-3.851	0.000	0.006	3A	Myb-related protein Hv33	Myb_DNA-binding
TRITD3Av1G014570	53.328	-1.362	0.316	-4.313	0.000	0.002	3A	Disease resistance protein RPM1	NB-ARC
TRITD3Av1G024490	3338.291	-0.880	0.228	-3.857	0.000	0.006	3A	NB-ARC domain	NB-ARC
TRITD3Av1G000840	231.624	1.596	0.420	3.796	0.000	0.007	3A	Cytochrome P450 89A9	p450
TRITD3Av1G020750	45.358	2.012	0.621	3.239	0.001	0.025	3A	Premnaspirodiene oxygenase	p450
TRITD3Av1G047940	240.718	-0.641	0.222	-2.888	0.004	0.049	3A	Serine carboxypeptidase II-1	Peptidase_S10
TRITD3Av1G187020	9.842	-2.377	0.736	-3.229	0.001	0.025	3A	Serine carboxypeptidase-like 18	Peptidase_S10
TRITD3Av1G204500	604.245	-1.222	0.267	-4.578	0.000	0.001	3A	Serine carboxypeptidase-like 18	Peptidase_S10
TRITD3Av1G214780	26.975	-1.402	0.451	-3.105	0.002	0.032	3A	Serine carboxypeptidase-like 27	Peptidase_S10

Table 3.8 continued

TRITD3Av1G162110	129.890	-0.775	0.206	-3.764	0.000	0.007	3A	Carboxyl-terminal-processing peptidase 1, chloroplastic	Peptidase_S41
TRITD3Av1G195240	56.899	-1.454	0.479	-3.037	0.002	0.037	3A	Subtilisin-like protease SBT2.2	Peptidase_S8
TRITD3Av1G278770	922.854	-1.515	0.371	-4.078	0.000	0.003	3A	Photosystem II D2 protein	Photo_RC
TRITD3Av1G278780	49.299	-1.491	0.442	-3.372	0.001	0.018	3A	Photosystem II D2 protein	Photo_RC
TRITD3Av1G091250	17.074	3.281	0.691	4.746	0.000	0.000	3A	Phosphatidylinositol 4-kinase gamma 1	PI3_PI4_kinase
TRITD3Av1G026310	1027.194	-0.433	0.127	-3.409	0.001	0.017	3A	Protein NSP-INTERACTING KINASE 3	Pkinase
TRITD3Av1G049310	1167.219	0.338	0.102	3.301	0.001	0.022	3A	Probable serine/threonine-protein kinase At1g09600	Pkinase
TRITD3Av1G074500	47.369	-2.868	0.456	-6.295	0.000	0.000	3A	Receptor-like serine/threonine-protein kinase At1g78530	Pkinase
TRITD3Av1G132870	611.760	-0.939	0.281	-3.341	0.001	0.020	3A	U-box domain-containing protein 34	Pkinase
TRITD3Av1G162170	51.567	-1.603	0.536	-2.992	0.003	0.040	3A	Probable receptor-like protein kinase At2g42960	Pkinase

Table 3.8 continued

TRITD3Av1G165370	47.776	-1.634	0.447	-3.656	0.000	0.009	3A	Putative receptor protein kinase ZmPK1	Pkinase
TRITD3Av1G210150	395.089	1.361	0.426	3.196	0.001	0.027	3A	Probable LRR receptor-like serine/threonine-protein kinase At1g06840	Pkinase
TRITD3Av1G214640	479.176	-0.416	0.142	-2.938	0.003	0.045	3A	Calcium-dependent protein kinase 3	Pkinase
TRITD3Av1G219280	321.944	-0.889	0.302	-2.940	0.003	0.044	3A	Serine/threonine-protein kinase Nek5	Pkinase
TRITD3Av1G235790	8.308	1.638	0.518	3.159	0.002	0.029	3A	Proline-rich receptor-like protein kinase PERK13	Pkinase
TRITD3Av1G102530	650.659	-1.115	0.336	-3.318	0.001	0.021	3A	Pto-interacting protein 1	Pkinase_Tyr
TRITD3Av1G181840	300.409	-0.527	0.119	-4.431	0.000	0.001	3A	Serine/threonine-protein kinase HT1	Pkinase_Tyr
TRITD3Av1G287490	726.906	0.954	0.287	3.318	0.001	0.021	3A	Probable LRR receptor-like serine/threonine-protein kinase At1g67720	Pkinase_Tyr
TRITD3Av1G133180	1910.369	-1.240	0.391	-3.170	0.002	0.028	3A	Probable protein phosphatase 2C 6	PP2C
TRITD3Av1G166900	1889.291	-2.061	0.662	-3.115	0.002	0.032	3A	Probable protein phosphatase 2C 50	PP2C

Table 3.8 continued

TRITD3Av1G003450	283.199	-0.955	0.293	-3.264	0.001	0.023	3A	Peptidyl-prolyl cis-trans isomerase CYP26-2, chloroplastic	Pro_isomerase
TRITD3Av1G213400	248.751	0.453	0.149	3.047	0.002	0.036	3A	Sorting nexin 2A	PX
TRITD3Av1G232720	24.405	-1.977	0.679	-2.913	0.004	0.047	3A	Ribonuclease 2	Ribonuclease_T2
TRITD3Av1G181880	95.369	-2.518	0.748	-3.365	0.001	0.019	3A	Carotenoid cleavage dioxygenase 8 homolog B, chloroplastic	RPE65
TRITD3Av1G229290	1413.343	0.474	0.120	3.943	0.000	0.004	3A	Serine hydroxymethyltransferase 7	SHMT
TRITD3Av1G248050	520.848	0.419	0.137	3.049	0.002	0.036	3A	Transcription factor TCP20	TCP
TRITD3Av1G182540	115.359	-2.145	0.496	-4.320	0.000	0.002	3A	Probable glutamate carboxypeptidase 2	TFR_dimer
TRITD3Av1G183180	1969.876	-0.810	0.208	-3.891	0.000	0.005	3A	Probable glutamate carboxypeptidase 2	TFR_dimer
TRITD3Av1G043260	164.484	1.453	0.479	3.035	0.002	0.037	3A	UDP-glycosyltransferase 73C3	UDPGT
TRITD3Av1G183980	130.511	-1.598	0.430	-3.715	0.000	0.008	3A	Anthocyanidin 5,3-O-glucosyl transferase	UDPGT
TRITD3Av1G184190	136.005	-2.329	0.335	-6.946	0.000	0.000	3A	Anthocyanidin 5,3-O-glucosyl transferase	UDPGT

Table 3.8 continued

TRITD3Av1G181020	797.108	0.275	0.084	3.269	0.001	0.023	3A	Ubiquitin-conjugating enzyme E2 36	UQ_con
TRITD3Av1G193550	301.312	-0.668	0.188	-3.547	0.000	0.012	3A	Hemimethylated DNA-binding protein YccV like	UVR
TRITD3Av1G169960	149.099	-0.531	0.164	-3.238	0.001	0.025	3A	Chloride channel protein CLC-e	Voltage_CLC
TRITD3Av1G074690	188.745	1.056	0.344	3.069	0.002	0.035	3A	WRKY transcription factor 6	WRKY
TRITD3Av1G184990	8.090	2.416	0.779	3.102	0.002	0.033	3A	Probable WRKY transcription factor 69	WRKY
TRITD3Av1G187430	1654.618	-0.712	0.240	-2.965	0.003	0.042	3A	E3 ubiquitin-protein ligase MIEL1	zf-CHY
TRITD3Av1G019850	1907.004	-0.460	0.137	-3.348	0.001	0.019	3A	E3 ubiquitin-protein ligase PRT6	zf-UBR1

Table 3.9. Durum wheat ANOVA results – significant metabolites from the complete dataset

Description	R.T	m/z	Formula	Stage F val	Stage adj p-val	Allele F val	Allele adj p-val	Interaction F val	Interaction adj p-val
linalool oxide D 3-[apiosyl-(1->6)-glucoside]	6.87	487.21	C21H36O11	5.42	0.44	8.85	0.01	1.16	0.74
cyanidin 3-rhamnoside	7.87	434.12	C21H21O10+	24.62	0.03	11.68	0.00	9.43	0.07
ptelatoside B	5.26	451.16	C20H28O10	2.27	0.53	16.93	0.00	3.72	0.29
sesaminol glucoside	5.22	555.15	C26H28O12	2.02	0.53	16.14	0.00	4.57	0.24
DIBOA-Glc	1.53	366.08	C14H17NO9	2.45	0.53	9.11	0.01	0.68	0.82
3,5-dihydroxyphenyl 1-O-(6-O-galloyl-β-D-glucopyranoside)	3.93	423.09	C19H20O12	2.44	0.53	5.45	0.04	0.16	0.97
niazimin A	6.31	406.15	C18H25NO8	1.69	0.56	5.26	0.04	0.93	0.76
cassitoroside	18.84	579.16	C25H32O14	0.16	0.83	19.13	0.00	1.56	0.60
taxifolin 3-arabinoside	0.86	437.11	C20H20O11	0.02	0.95	5.07	0.05	0.55	0.85
cholesteryl-β-D-glucoside	7.13	531.41	C33H56O6	4.90	0.49	21.95	0.00	3.44	0.32
rubrofusarin 6-[glucosyl-(1->3)-glucosyl-(1->6)-glucoside]	5.24	759.23	C33H42O20	2.01	0.53	6.65	0.02	1.46	0.62
dibutyl malate	5.57	229.14	C12H22O5	0.11	0.86	26.36	0.00	2.34	0.46
gibberellin A125	5.21	401.15	C20H26O7	0.01	0.96	15.71	0.00	0.92	0.76
(2E,4E,6E)-7-hydroxy-4-methylhepta-2,4,6-trienal	0.81	139.07	C8H10O2	0.87	0.66	7.83	0.01	1.24	0.73

Table 3.9 continued

4-coumaroyl-2-hydroxyputrescine	4.59	273.12	C13H18N2O3	0.70	0.66	7.02	0.02	1.69	0.56
reduced β-nicotinamide D-ribonucleotide	9.29	317.05	C11H15N2O8P-2	0.04	0.91	8.51	0.01	0.84	0.79
PE-NMe(16:0/18:1(9Z))	6.14	754.54	C40H78NO8P	2.08	0.53	18.07	0.00	4.20	0.26
PE-NMe(16:0/18:1(9Z))	5.84	732.55	C40H78NO8P	0.13	0.84	5.17	0.05	3.11	0.39
PE-NMe(16:0/16:0)	5.83	728.52	C38H76NO8P	0.01	0.95	5.96	0.03	2.53	0.45
lycopersiconol	6.84	335.26	C21H34O3	4.05	0.49	9.17	0.01	7.86	0.08
canarigenin 3-[glucosyl-(1->4)-6-deoxy-alloside]	4.86	663.33	C35H52O13	1.41	0.60	12.27	0.00	6.02	0.13
3,5-dihydroxy-6,7-didehydro-12'-apo-β-caroten-12'-al	6.78	431.25	C27H36O3	6.73	0.44	7.48	0.01	1.97	0.51
spinacoside D	6.13	765.40	C40H60O14	0.71	0.66	11.74	0.00	4.12	0.26
furohyperforin	8.40	553.39	C35H52O5	0.10	0.86	10.45	0.01	0.51	0.86
soyasaponin IV	5.81	749.45	C41H66O13	0.01	0.95	6.98	0.02	2.35	0.46
28-glucosyl-3b,23-dihydroxy-12,19(29)-ursadien-28-oate 3-arabinoside	5.83	747.43	C41H64O13	0.01	0.95	6.67	0.02	2.60	0.44
α-crocetin glucosyl ester	7.29	513.21	C26H34O9	2.13	0.53	8.75	0.01	1.89	0.54
(3xi,5Z)-1,5-octadien-3-ol	0.79	109.10	C8H14O	2.16	0.53	6.36	0.03	0.92	0.76
23-acetoxysoladulcidine	7.39	496.34	C29H47NO4	0.50	0.69	11.63	0.00	1.24	0.73
3-methyldioxyindole	4.87	146.06	C9H9NO2	0.70	0.66	6.84	0.02	2.13	0.48
2",4",6"-triacetylglycitin	7.09	555.15	C28H28O13	3.10	0.49	23.11	0.00	5.19	0.18

Table 3.9 continued

vestitone 7-glucoside	8.22	435.17	C22H26O9	0.69	0.66	8.27	0.01	0.81	0.79
procyanidin	5.10	579.14	C30H26O12	0.01	0.95	12.24	0.00	0.28	0.93
laudanosine	1.38	381.19	C21H28NO4+	0.50	0.69	8.68	0.01	5.94	0.13
(S)-valerianine	14.13	178.12	C11H15NO	3.41	0.49	23.11	0.00	1.92	0.53
2'-hydroxynicotine	6.24	161.11	C10H14N2O	0.07	0.88	8.11	0.01	8.41	0.07
2,3-dihydro-5-(5-methyl-2-furanyl)-1H-pyrrolizine	8.52	170.10	C12H13NO	4.02	0.49	7.93	0.01	1.16	0.74
1-(2H-1,3-benzodioxol-5-yl)-2-[2,6-dimethoxy-4-(prop-2-en-1-yl)phenoxy]propyl acetate	5.27	437.15	C23H26O7	0.77	0.66	7.76	0.01	6.94	0.10
4',5-dihydroxy-3,3'-dimethoxy-6,7-methylenedioxyflavone 4'-glucuronide	0.86	557.10	C24H22O14	1.10	0.63	6.89	0.02	1.37	0.66
piperundecalidine	6.44	390.20	C23H29NO3	0.31	0.75	5.32	0.04	1.65	0.58
cohibin C	5.81	599.50	C37H68O4	0.50	0.69	7.95	0.01	1.78	0.56
neoreticulatacin A	6.85	575.50	C37H68O5	2.85	0.52	5.40	0.04	3.62	0.30
montecristin	5.83	597.49	C37H66O4	0.00	0.97	5.96	0.03	2.94	0.39
dieporeticenin	5.59	595.47	C37H64O4	0.49	0.69	5.69	0.04	2.53	0.45
annonaceous acetogenin	6.18	573.49	C35H66O4	1.29	0.61	11.18	0.00	4.29	0.26
annonaceous acetogenin	5.83	575.50	C37H68O5	0.33	0.75	6.83	0.02	2.08	0.49
2-methylbutylamine	0.81	110.09	C5H13N	0.21	0.81	5.45	0.04	0.98	0.76
14α-hydroxypaxilline	7.53	434.23	C27H33NO5	0.33	0.75	6.28	0.03	0.36	0.90

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CHAPTER FOUR

COMPARISON OF SPECTRAL REFLECTANCE, PRIMARY PHYSIOLOGY AND VOLATILE ORGANIC COMPOUNDS IN NEAR ISOGENIC LINES OF SPRING AND DURUM WHEAT ASSOCIATED WITH EARLY STEM SOLIDNESS AND RESISTANCE TO WHEAT STEM SAWFLY

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Manuscript Information Page

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BMC Plant Biology
Status of Manuscript:
_X_ Prepared for submission to a peer-reviewed journal
___Officially submitted to a peer-review journal
___ Accepted by a peer-reviewed journal
___ Published in a peer-reviewed journal

Abstract

Substantial yield loss due to damage by the wheat stem sawfly (WSS) is a growing problem in North America, highlighting a need to explore the mechanisms that may be responsible for resistant phenotypes. The early stem solidness phenotype, associated with the *Qss.msub-3BLc* allele in spring wheat and the *Qss.msub-3ALb* allele in durum wheat, provides the plant with solid stems early in plant development, which coincides with the time of flight and oviposition period of WSS. In these cultivars, pith is reabsorbed as the plant matures which allows resources to be re-allocated during grain fill and may also support parasitoid populations that can be negatively affected by the use of solid stemmed cultivars. Here, we collected information on spectral reflectance and primary and secondary physiology to identify associations with the early stem solidness phenotype in spring and durum wheat at Zadoks 32 and Zadoks 49. Our results indicate that WSS likely use a combination of visual, olfactory and temperature cues to locate host plants. Photosynthetic parameters including photosynthetic rate, transpiration rate, conductance and intercellular CO2 decreased as spring wheat lines matured, while these parameters increased with maturity in durum wheat lines. For both spring and durum wheat, principal component analysis identified large values for wavelengths between 720 and 1350nm and small values for wavelengths between 350 and 400 nm as important for discriminating between early and late growth stages. Z-3-hexenyl acetate, nonanal and decanal showed significant differences between growth stages in both wheat species. These compounds have the potential to prime neighboring wheat plants for defense against infestation and can also induce production of extrafloral nectar which can increase longevity and fecundity in parasitoids of WSS. The early stem solidness alleles have different effects on flag leaf reflectance,

photosynthetic parameters and volatile emissions in spring and durum wheat near isogenic lines which imply both direct and indirect effects on the behavior of WSS or their parasitoids.

Introduction

Wheat stem sawfly, *Cephus cinctus* Norton, is the most economically damaging pest to dryland wheat production in the North American Great Plains. This native species has adapted from host prairie grasses to cause damaging populations in cultivated cereals in a sequence beginning with spring wheat, followed by winter wheat, and most recently - barley (Achhami et al., 2020; Ainslie, 1929; Lesieur et al., 2016; Morrill and Kushnak, 1996; Varella et al., 2018, Weaver, 2023; Weiss and Morrill, 1992). Emergence of adult WSS is synchronized with the stem elongation stage in spring bread wheat (*Triticum aestivum* L.) and spring durum wheat (*Triticum turgidum* L. var *durum*), making these cereals prime targets for oviposition by female WSS (Holmes & Peterson, 1960). Eggs are deposited inside the stem and after hatching, the larvae feed on parenchymal tissue lining the stem until the plant ripens, at which point the larvae will travel to the base of the stem and girdle the inside at soil level, weakening the stem. Both photosynthetic ability and resulting head weight are negatively affected by the damage to vascular tissues that is caused by larval feeding, and the stem girdling activity of mature larvae weakens stems, increasing susceptibility to lodging and making it difficult to recover heads during harvest (Ainslie, 1920; Delaney et al., 2010; Macedo et al., 2005; Morrill et al., 1998; Sherman et al., 2010). WSS is difficult to control due to the fact that larvae are protected inside the stem and the long duration of adult emergence (Beres et al., 2011; Knodel et al., 2009). The most frequently used method of management adopted by producers is the use of solid stemmed cultivars, which are thought to inhibit the growth, development, and survival of WSS larvae

(Bathini et al., 2023; Delaney et al., 2010; Holmes & Peterson, 1961, 1962; Peirce et al., 2022). While somewhat effective, the expression of the stem solidness phenotype can be highly variable depending on environmental effects such as light quality, sowing density and water availability (Beres et al., 2017; Nilsen, 2017; Nilsen et al., 2016; Roemhild, 1954). There have been historic and ongoing concerns that the solid stem phenotype may be associated with lower yields in some genetic backgrounds, although modern solid stem cultivars often outperform their hollow stemmed counterparts, especially under drought conditions or when populations of WSS are large (Hayat et al., 1995; Lebsock & Koch, 1968; McNeal & Berg, 1979; McNeal et al., 1965; Saint Pierre et al., 2010; Wallace & McNeal, 1966). Biological control in the form of parasitism by the braconid wasps *Bracon cephi* (Gahan) and *Bracon lissogaster* Muesebeck can also be hindered by the use of solid stemmed cultivars (Rand et al., 2020; Rand et al., 2012; Runyon, 2001; Wu et al., 2013). For these reasons, interest has grown in the early stem solidness phenotype first observed in spring wheat cultivar Conan. With this phenotype, stems are filled with pith early in plant development which disappears as the plant matures, providing protection to the plant during the critical flight period of WSS and allowing resources from pith to be reallocated during the grain filling stage (Varella et al., 2016; Varella et al., 2017; Varella et al., 2019b).

In spring wheat, the stem solidness trait is associated with the quantitative trait locus (QTL) *Qss.msub-3BL*, and several alleles that confer differing levels of stem solidness have been identified at this locus (Cook et al., 2019; Cook et al., 2004; Varella et al., 2017; Wong et al., 2023). The hollow stemmed phenotype in spring wheat is associated with the allele *Qss.msub-3BLa*, while *Qss.msub-3BLc* is an allele for early stem solidness that was identified in the

cultivar Conan (Varella et al., 2016). An allele for early stem solidness in durum wheat has also been identified on chromosome 3A, designated *Qss.msub-3ALb* (Varella et al., 2019b). In spring wheat, the early stem solidness allele *3BLc* significantly decreases the number of ovipositor insertions and number of eggs laid by WSS females, which leads to overall lower rates of stem cutting while early solid durum wheat lines with the *3ALb* allele experience reduced rates of stem cutting due to increased larval mortality (Varella et al., 2016; Varella et al., 2017). Associations between early stem solidness, reflectance characteristics and primary and secondary metabolic processes may help explain the similarities in plant response to WSS infestation in early solid spring and durum wheat cultivars.

Volatile organic compounds (VOCs) are secondary metabolites, the precursors of which originate from primary metabolic processes (Picazo-Aragonés et al., 2020). VOCs are associated with plant resistance to insect pests in many ways since they not only influence the behavior of many pest species, including wheat stem sawfly, but they are also induced by insect feeding and can influence both defense responses of nearby plants and attract natural enemies of pest species (Ameye et al., 2015; Ameye et al., 2020; Clavijo-McCormick et al., 2012; Ninkovic et al., 2021). Z-3-hexenyl acetate, Z-3-hexanol, E-β-ocimene, E-2-hexenal and β-caryophyllene make up the majority of volatile compounds emitted by wheat (Buttery et al., 1985). Several of these common wheat VOCs have been identified as potential attractants to female WSS including Z-3-hexenyl acetate, β-ocimene and Z-3-hexen-1-ol (Piesik et al., 2008; Weaver et al., 2009).

The spring wheat cultivars Reeder and Conan differ in terms of host plant attractiveness to WSS, with ovipositing females preferring volatile blends released by Reeder over Conan (Varella et al., 2017; Weaver et al., 2009). Conan is known to emit significantly lower amounts

of Z-3-hexenyl acetate compared to Reeder, a phenomenon also observed in resistant cultivars of winter wheat (Buteler & Weaver, 2012; Buteler et al., 2010; Weaver et al., 2009). Conan also experiences less infestation and has lower rates of parasitism than hollow stemmed cultivars (Talbert et al., 2014). While a previous study using spring wheat near isogenic lines (NILs) suggests that the *3BLc* allele does not appear related to volatile blends in terms of attracting females to the plant, NILs with the early stem solidness allele *3BLc* still experienced fewer ovipositor insertions by foraging females and less overall oviposition than NILs with the *3BLa* hollow stemmed allele (Varella et al., 2017). While volatile compounds, host plant attractiveness and rates of parasitism have not been reported for wheat stem sawfly interactions with durum wheat, early stem solid cultivar Pierce experiences lower rates of stem cutting than other landrace accessions of durum, even when infestation levels are high (Varella et al., 2019b). It is possible that in spring and durum wheat, volatile compounds or contact with other compounds on the plant surface are influencing behavior of WSS females or even attracting parasitoids *B. cephi* and *B. lissogaster*, causing increased mortality (Lavergne et al., 2018; Varella et al., 2017). Quantifying differences in the composition of the volatile profiles from uninfested NILs of spring and durum wheat at different growth stages will help determine whether volatile compounds govern oviposition-related behavior of female WSS and whether these differences are associated with the early stem solidness trait.

Several studies have identified the effects of wheat stem sawfly infestation and subsequent larval feeding on primary physiology, but so far none have explored differences in photosynthetic parameters related to early stem solidness or inherent resistance to WSS (Delaney et al., 2010; Lavergne et al., 2020; Macedo et al., 2005; Macedo et al., 2006, 2007). In wheat,

photosynthetic capacity varies significantly depending on cultivar, with the highest yielding cultivars often exhibiting increased maximum photosynthetic rates, carbon exchange rates and stomatal conductance especially at the grain filling stage (Blum, 1990; Driever et al., 2014; Fischer et al., 1998; Furbank et al., 2013). Results from previous time course studies of wheat plants infested by WSS indicated that infestation can have a significant negative effect on gas exchange rates and photosynthetic efficiency, especially under drought stress or nutrient deficiency, although any negative effects on photosynthesis due to larval feeding might be offset by increased photochemical efficiency of photosystem II in developing wheat heads, with solid stemmed cultivars appearing better able to compensate for damage from larval feeding (Delaney et al., 2010; Macedo et al., 2005; Macedo et al., 2006, 2007). This suggests the possibility that primary metabolism is different in resistant cultivars with early stem solidness compared to hollow stemmed susceptible cultivars even in the absence of sawfly infestation. By measuring photosynthetic parameters in resistant and susceptible lines we can determine whether there are inherent differences between hollow and early solid lines in the absence of infestation that may allow plants to better protect themselves against potential oviposition by WSS females or herbivory by WSS larvae.

The structure and chemical components of leaves dictate how light energy is absorbed and scattered (Gates et al., 1965). Cellulose in cell walls, air spaces between cells, leaf water content, water soluble compounds and pigments such as chlorophyll can all be affected by biotic and abiotic stresses which in turn has an effect on reflectance (Gates et al., 1965; Hunt & Rock, 1989). Many mono or oligophagous insect species use visual cues such as reflectance in combination with olfactory and direct chemical perception to locate host plants including apple

maggot *Rhagoletis pomonella* Walsh, apple sawfly *Hoplocampa testudinea* and emerald ash borer *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) (Aluja & Prokopy, 1993; Crook et al., 2009; Owens, 1982; Prokopy & Owens, 1978). There is also strong evidence that several species of Hymenopteran braconid parasitoids prefer certain hues, indicating that the reflectance of plants is involved in attracting natural enemies even in the absence of their target pest species (Cooperband et al., 2013; Messing & Jang, 1992; Pérez et al., 2012). While reflectance spectroscopy is generally used to determine plant health and physiological status in response to environmental stress, characterizing the spectral signatures of hollow stemmed lines and lines with the early stem solidness phenotype will help determine whether visual or hyperspectral cues could also be utilized by foraging WSS.

The relationship between primary metabolism, flag leaf reflectance, plant volatile production and the early stem solidness phenotype has not yet been explored in paired NILs, but this can provide valuable information about host plant attractiveness to foraging WSS. Here, we utilize an analytical spectral device (ASD), portable gas exchange system (LI-6400) and volatile collection system (VCS) to compare spectral reflectance, photosynthetic parameters and volatile organic compounds (VOCs) in nearly isogenic pairs of durum and spring wheat containing different alleles for stem solidness at the 3AL and 3BL QTLs. We connect spectral signatures with measurements of photosynthetic capacity and emissions of volatile compounds to identify potential mechanisms influencing foraging and oviposition behaviors of WSS and resistance to infestation associated with early stem solidness.

Materials and Methods

Plant Culture.

The spring wheat breeding program at Montana State University developed the near isogenic lines (NILs) used in this study. NILs were developed from RIL populations which used spring wheat parents Reeder and Conan and durum wheat parents Pierce and SD9 (PI 41353) were chosen for their differing expression of the solid stem phenotype. Reeder contains the *Qss.msub-3BLa* allele, which is associated with the hollow stem phenotype, while Conan exhibits early stem solidness associated with the *Qss.msub-3BLc* allele. One NIL pair for the 3B QTL consisting of a resistant line with the *Qss.msub-3BLc* allele which confers early stem solidness and a susceptible line with allele *Qss.msub-3BLa* which exhibits a hollow stemmed phenotype was used. The durum NIL pair consisted of a resistant line with the *Qss.msub-3BLb* allele which is associated with the early stem solidness phenotype, and a susceptible line with the allele *Qss.msub-3BLa.*

Wheat plants were grown from seed at the Montana State University Plant Growth Center in Bozeman, Montana. Individual seeds were sown in conical pots containing 50:50 mix of PGC Soil Mix (Bozeman Silt Loam Soil, Washed Concrete Sand, and Canadian Sphagnum Peat Moss) and Sunshine Mix #1 (Canadian Sphagnum Peat Moss, perlite, vermiculite, and Dolomitic lime). Plants were maintained under greenhouse conditions (22℃ ± 2℃ during the day and 20℃ ± 2℃ during the night, and photoperiod of 15L:9D h). with natural and artificial light (GE Multivapor lamps; model MVR1000/C/U, GE Lighting, General Electric Co., Cleveland, Ohio). Plants were watered daily and fertilized once a week with Peters Professional® General Purpose

Fertilizer (J.R. Peters, Inc., Allentown, Pennsylvania, United States) at 100 ppm in aqueous solution.

Spectral Reflectance Data Collection.

At the start of stem elongation (approximately Zadoks stage 34), and again when the first awns became visible (Zadoks stage 49) six plants were randomly chosen from the NIL populations containing the early stem solidness allele from Conan, *3BLc* and a hollow stem allele from Reeder, *3BLa*. Spectral data was collected in the greenhouse from the adaxial surface of the third leaf of the primary stem of plants at Zadoks 32 and from the adaxial surface of the flag leaf of plants at Zadoks 49 using an Analytical Spectral Devices (ASD) FieldSpec® Pro spectroradiometer (Malvern Panalytical, Malvern UK, formerly Analytical Spectral Devices). A Spectralon® standard white reference panel (Labsphere, North Sutton, New Hampshire) was used to calibrate the sensor prior to data collection. The spectral resolution was 3 nm in the 350 to 1050 nm range, and 10 nm in the 1051-2500 nm range. Two spectra per plant were collected and averaged to obtain a mean spectral reflectance for each plant.

Primary Physiology Measurements.

Immediately after collection of spectral data, measurements of photosynthetic rate (P_s), transpiration rate (E), stomatal conductance (g_s) and intercellular CO_2 (C_i) were recorded from the third leaf of the primary stem of plants at Zadoks 32 and the flag leaf of the primary stem of plants at Zadoks 49 using a portable photosynthesis system (model LI-6400; LI-COR, Lincoln, Nebraska, USA). Leaves were placed into a 2-cm^2 chamber and gas exchange parameters were recorded at a light intensity of 1,200 µmol photons/m^2/s and a CO2 concentration of 400

µmol/mol under constant flow of 500 µmol/s. Plants were then allowed to rest for 24 hours before the collection of volatile organic compounds. This process was repeated for six replicates.

Collection of Volatile Compound Profiles.

The collection of the volatile organic compounds (VOCs) took place in a greenhouse environment at the MSU Plant Growth Center, Bozeman, Montana. The protocol used was as described by Piesik et al. (2006), where the main stems of individual plants were enclosed inside a glass volatile collection chamber (4cm in diameter, 80 cm long). The upper part of the chamber was attached to a volatile collection port, where a glass volatile collection trap was inserted (6.35mm diameter, 76mm long; Analytical Research Systems, Gainesville, Florida) with 30mg of Super-Q adsorbent (Alltech Associates, Deerfield, Illinois). To maintain the pressure and flow rate in each glass chamber, a regulated vacuum pump supplied purified and humidified air at a rate of 1.0 liter/min. At the base of the chamber a Teflon guillotine was used to tightly enclose the plant stem and prevent outside air and extraneous volatiles from soil from entering the system.

After collecting VOCs for 6h (1000-1600h), traps were flash eluted using 200 µL of methylene chloride via compressed nitrogen gas (99.9%) and using (Fisher Scientific, Fair Lawn, NJ). The extract was collected in a 0.2 mL conical glass insert placed in a 12 x 32 vial (SUN-SRi Chromatography Accessories, Rockwood, Tennessee, United States) inserted into a 1.5 mL clear screw cap vial (Agilent Technologies, Santa Clara, California, United States), and 10 µL of a 30 ng μL^{-1} solution of *n*-nonyl acetate (Sigma-Aldrich, Saint Louis, MO) in methylene chloride was added as an internal standard. Samples were analyzed using gas chromatography-mass spectrometry (GC-MS; Agilent 6890 instrument, Agilent Technologies, Santa Clara, California)

fitted with an HP-5MS column (30 m x 0.25 mm, 0.25 µm film thickness; J&W Scientific, Folsom, CA). The oven profile consisted of an initial temperature of 50 °C followed by a ramp of 5 °C min^{-1} to 200 °C, then a second ramp of 25 °C min^{-1} to 275 °C.

Volatile compounds were identified by comparing retention times and mass spectra with commercial standards using ChemStation® software (Agilent Technologies, Wilmington, DE) and the NIST Mass Spectral Library (National Institute of Standards and Technology, Gaithersburg, MD). Compounds with a matching score >900 in the NIST library were used for analysis. The quantification of compounds was standardized for the number of hours for which they were collected and for aboveground plant biomass (ng g^{-1} h^{-1}).

Analysis of Primary Physiology Measurements.

Photosynthetic response data collected with the portable photosynthesis system was analyzed using a multivariate analysis of variance (MANOVA) in R (MANOVA; α=0.05; R Core Team, 2022). Means were compared using a univariate one-way ANOVA to identify which photosynthetic parameters were responsible for significant differences.

Analysis of Spectral Reflectance Data.

Spectral data were averaged to obtain a mean spectral signature for each of the near isogenic lines at both growth stages. Principal components analysis (PCA) was performed on raw spectral data using base R and the ggplot2 package was utilized for visualization (R Core Team, 2022, Wickham 2016).

Analysis of Volatile Compound Profiles.

Volatile compound data was square root transformed and analyzed using a multivariate analysis of variance (MANOVA) in R (MANOVA; α=0.05; R Core Team, 2022). Means were compared using a univariate one-way ANOVA to determine volatile compounds that were quantitatively different.

Results

Spectral Reflectance.

At Zadoks stage 32, spring wheat NILs containing the *3BLa* allele had noticeably greater reflectance than NILs containing the *3BLc* allele in the 700-1350nm range (Figure 4.1a), while at Zadoks 49 the opposite trend was observed, with noticeably greater reflectance observed in *3BLc* NILs compared to *3BLa* NILs (Figure 4.1b). Both NILs with the *3BLc* allele and NILs with the *3BLa* allele had noticeably increased reflectance at Zadoks 49 in the 700-2500nm range but NILs with the *3BLc* allele had lower reflectance between 300 and 600 nm as they matured (Figure 4.1c). Reflectance decreased with maturity between a greater range of 300 and 1350 nm in NILs with the *3BLa* allele. (Figure 4.1d). Principal components analysis of the spring wheat NILs was able to discriminate between growth stages (Figure 4.2). The first principal component explained 68.08% of the variance in the dataset and was defined by large values in the 740-1300nm range and small values for wavelengths above 1870 nm and between 350 and 410 nm. The second principal component explained 11.90% of variance and was defined by small values between 720 and 1100nm and large values between 1370 and 1870nm.

Durum wheat NILs containing the *3ALb* allele had noticeably greater reflectance in the 700-1350 nm range and the 1550-1850nm range (Figure 4.3d). The same trend was observed for

NILs containing the *3ALa* allele (Figure 4.3c). The spectral signatures for NILs with the *3ALb* and *3ALa* alleles were nearly identical at both Zadoks 32 and Zadoks 49, although *3BLb* lines had slightly higher reflectance in the 700-1350nm range (Figure 4.3a-b). Principal components analysis was able to discriminate between growth stages, although data collected at Zadoks 32 showed greater spread than those collected at Zadoks 49 (Figure 4.4). The first principal component explained 79.23% of the variability in the dataset and was defined by large values between 720 and 1350 nm and small values for wavelengths between 350 and 375nm and above 1870nm, while the second principal component explained 16.67% of the variability and was defined by small values in the 735 to 1500 nm range and large values above 1400nm.

Primary Physiology.

Growth stage and temporal replicate both had a significant effect on the photosynthetic parameters of spring wheat NILs, with no overall significant effect of allele (Table 4.1). Allele and temporal replicate had a significant effect on photosynthetic rate (p-value <0.05), while growth stage and temporal replicate had a significant effect on intercellular CO2 (p-values<0.05) and transpiration rate (p-values<0.05)(Table 4.2). The photosynthetic rate was significantly higher in resistant lines with the *3BLc* allele, with no change between growth stages observed for either allele (Figure 4.5a). Transpiration rate and intercellular CO2 were significantly lower at Zadoks 49 in both lines (Figure 4.5b-c). Temporal replicate had a significant effect on conductance (p-value<0.05), but while conductance was lower at Zadoks 49, this difference between growth stages was not found to be significant (p-value=0.06, Figure 4.5d). There was no evidence for any interactions between variables.

There was also a significant effect of growth stage and temporal replicate on the photosynthetic parameters in durum wheat NILs, with no overall significant effect of allele (Table 4.3). Growth stage and temporal replicate had a significant effect on the photosynthetic rate, intercellular CO2 and transpiration rate (p-values<0.05), while only temporal replicate had an effect conductance (p-value<0.05)(Table 4.4). Photosynthetic rate and transpiration rate were significantly higher at the later growth stage regardless of allele (Figure 4.6a-b), while intercellular CO2 was significantly lower (Figure 4.6c). There was no evidence for any interactions between the variables.

Volatile Profiles.

In spring wheat NILs, growth stage had an overall significant effect on volatile compound abundance (p-value<0.05) with no effect of allele or temporal replicate and no evidence for any interactions between compounds (p-value>0.05, Table 4.5). Growth stage had an effect on the abundance of Z-3-hexenyl acetate, β-linalool, nonanal, α-terpineol, decanal and caryophyllene (p-value<0.05, Table 4.6). All compounds except α-terpineol were significantly lower at Zadoks 49 (Figure 4.7). Z-3-hexenyl acetate was higher in NILs with the *3BLc* allele, but this difference was not found to be significant (p-value=0.05).

In durum wheat NILs, growth stage had an overall significant effect on the abundance of volatile compounds (p-value<0.05), with no effect of allele or temporal replicate and no evidence for interactions between any of the variables (p-value>0.05, Table 4.7). Allele and growth stage had a significant effect on the abundance of Z-3-hexenyl acetate and caryophyllene (p-value <0.05 for both). Z-3-hexenyl acetate was significantly higher at Zadoks 49, particularly in susceptible lines (Figure 4.8a, Table 4.8). Caryophyllene was lower in early solid lines with the

3ALb allele and showed a significant decrease in both lines at the later growth stage (Figure 4.8f, Table 4.8). Stage and temporal replicate both had a significant effect on the abundance of nonanal, which increased at Zadoks 49 in both lines (p-value<0.05, Figure 4.8c, Table 4.8), with evidence for an interaction between allele and growth stage (p-value<0.05). α-terpineol was significantly affected by allele and temporal replicate (p-value<0.05), with a significant interaction between allele and temporal replicate (p-value<0.05) as well as growth stage and temporal replicate (p-value<0.05, Table 4.8). There was a significant effect of growth stage on the abundance of decanal, with increased abundance observed at Zadoks 49 (p-value<0.05, Figure 8e, Table 4.8). There was no effect of allele or growth stage on the abundance of β-linalool (p-value>0.05, Table 4.8).

Discussion

Oligophagous insects like WSS are thought to use visual cues in combination with volatile compounds and contact stimuli to locate and choose a host (Crook et al., 2009; Prokopy & Owens, 1978). For example, apple maggot *Rhagoletis pomonella* Walsh is attracted to colors whose reflectance closely mimics that of foliage as well as red hues that are similar to ripe apples. However, when color is not detectable females will instead rely on olfactory cues to seek out and oviposit in fruits regardless of color or ripeness (Aluja & Prokopy, 1993; Owens, 1982). Visual cues are also used by natural enemies to locate host plants of their target pest species. While color and reflectance preferences for the WSS parasitoids *B. cephi* and *B. lissogaster* have not been determined, several other species of braconid parasitoids are attracted to dark yellow hues with higher reflectance values between 550 and 630 nm (Cooperband et al., 2013; Messing & Jang, 1992; Pérez et al., 2012).

The majority of insect species are trichromats, utilizing three channels to view their environment and distinguish between colors (Lebhardt & Desplan, 2017; Peitsch et al., 1992). While not much is known about WSS vision or that of the obligate specialist braconid parasitoids, the eyes of most other Hymenopteran species contain UV, blue and green spectral receptor types which have sensitivity maxima around 340nm, 430nm and 535nm respectively. This means that WSS and their braconid parasitoids *B. cephi* and *B. lissogaster* are likely able to perceive wavelengths in the visible spectrum between 300 and 700nm. As plants matured, early solid spring wheat lines with the *3BLc* allele had lower reflectance in the visible range, while hollow stemmed lines with the *3BLa* allele had lower reflectance between a greater range of 300 and 1350 nm, just outside the visible range. Small values for wavelengths within the visible range (350 and 410 nm) also defined the first principal component, indicating its importance in discriminating between growth stages. Spectral differences in spring wheat cultivars with the *3BLc* allele from Conan may be related to the high degree of glaucousness observed in Conan compared to other cultivars of spring and winter wheat (Lavergne et al., 2018). Additionally, Conan has higher water content at Zadoks 32 compared to other hollow stemmed cultivars, which may explain the higher reflectance observed at the early growth stage in lines with the *3BLc* allele in our results as water loss commonly leads to an overall decrease in spectral signature (Hunt & Rock, 1989; Varella et al., 2016). Reflectance of the durum wheat lines did not appear to differ by growth stage in the visible range however wavelengths between 350 and 375nm did allow for discrimination between growth stages by principal component analysis. These results indicate that WSS might benefit from detecting reflectance in the visible spectrum in spring wheat, since reflectance appears to be higher in susceptible lines with the *3BLa* allele,

while in durum wheat lines, visual detection of suitable hosts seems less likely. This also suggests that parasitoids of WSS may be better able to locate WSS in spring wheat via visual cues. Wavelengths in the near infrared region from 750-2500 nm (NIR) are also perceived by insects as heat energy, often via gustatory reception (GR) (Iwase et al., 2023; Montell, 2013; Tsai et al., 2020). Although the functions have not been characterized, the WSS genome contains 35 GR genes (Robertson et al., 2018), so it is possible that they are also able to recognize and respond to wavelengths in the NIR. In the principal component analysis of durum wheat lines, the first principal component was defined by large values between 720 and 1350 nm, meaning that in durum wheat reflectance at these wavelengths may affect the behavior of WSS adults in field conditions by influencing leaf or canopy temperature.

Reflectance has also been used for predicting rates of photosynthesis, transpiration and emission of some volatile compounds including isoprene and other monoterpenes (Dechant et al., 2017; Heckmann et al., 2017; Penuelas et al., 2013; Sellers, 1985; Sellers et al., 1992). Spectral methods at the leaf level are most often used to estimate concentrations of leaf compounds such as chlorophyll to determine photosynthetic capacity (Hallik et al., 2017; Tian et al., 2019). However, these methods can also be applied to the plant canopy relationships between reflectance, photosynthetic rate and VOC emissions (Dechant et al., 2017; Heckmann et al., 2017; Penuelas et al., 2013; Sellers et al., 1992). Genotype effects on reflectance and headspace composition can also be observed in some plant species (Palmqvist et al., 2021).

WSS have many odorant receptor genes which are present mostly as single genes or small clusters, and while the specific functions of these genes has not been characterized, behavioral studies reveal that adult sawflies are able to detect and respond to specific volatile

compounds that are emitted by several host species (Robertson et al., 2018). WSS females are attracted to the common wheat green leaf volatile (GLV) compounds Z-3-hexenyl acetate, β-ocimene and Z-3-hexen-1-ol, however the relationship between emission of these compounds and plant resistance appears to differ depending on species (Piesik et al., 2008). The highly susceptible winter wheat cultivar Norstar has been found to emit more E- and Z- β-ocimene than resistant cultivars, while susceptible spring wheat cultivar Reeder releases greater amounts of Z-3-hexenyl acetate and resistant barley cultivar Craft emits more linalool and less Z-3-hexenyl acetate than susceptible cultivar Hockett (Achhami et al., 2021; Buteler et al., 2010; Weaver et al., 2009). This illustrates that while these compounds are perceived by WSS and are known to influence their behavior, concentration and/or ratio of these with other volatile compounds in the overall blend is also important. The effects of total concentration of volatiles and the ratio of specific behaviorally active compounds have also been observed in many other plant-insect systems (Bruce et al., 2005; Cha et al., 2011).

Volatile compounds were previously identified from spring wheat cultivars Reeder and Conan which contain the *3BLa* and *3BLc* alleles respectively. The susceptible cultivar Reeder was shown to emit greater amounts of Z-3-hexenyl acetate than early solid cultivar Conan, while our results showed that allele had no significant effect on emission of Z-3-hexenyl acetate. This suggests that alleles at the 3B locus are not associated with the concentration of Z-3-hexenyl acetate emitted by spring wheat. However, a significant effect of growth stage was observed in durum lines which was dependent on allele, indicating that the 3A locus may be involved in regulating emissions of Z-3-hexenyl acetate. Not only are WSS attracted to Z-3-hexenyl acetate, but there is also evidence that suggests that this compound may also attract some species of

braconid parasitoids, including both *B. cephi* and *B. lissogaster* (James, 2005; Pérez, 2009; Whitman & Eller, 1992). Some plant species also respond to Z-3-hexenyl acetate by increasing secretion of nectar from extrafloral nectaries, which provide foraging adult parasitoids with needed carbohydrates (Kost & Heil, 2006, 2008). Wheat plants do not have extrafloral nectaries, but many parasitoid species visit nearby plants to utilize carbohydrates from floral and extrafloral nectar for supplemental energy (Wäckers, 2005). *B. cephi* and *B. lissogaster* benefit from feeding from extrafloral nectaries in cowpea *Vigna unguiculata* (L.) Walpers and supplemental carbohydrates from extrafloral nectar increase egg load and volume, particularly in *B. cephi* females (Cavallini et al., 2022; Cavallini et al., 2023). Additionally, plants exposed to Z-3-hexenyl acetate experience an oxidative burst and activation of pathways related to antioxidative processes including changes to the phenylpropanoid pathway, meaning that emission of this compound may affect nearby plants and induce changes that enhance resistance to WSS feeding (Ameye et al., 2015; Ameye et al., 2020).

Greater emissions of linalool and caryophyllene are often induced in response to insect oviposition, feeding or mechanical damage to plant tissues (Piesik et al., 2022; Piesik et al., 2006; Sendel et al., 2022). Linalool induces cold stress tolerance in some plant species and along with Z-3-hexenyl acetate can also cause increased nectar production in extrafloral nectaries (Kost & Heil, 2006; Zhao et al., 2020). Therefore, in spring wheat, greater emission of β-linalool as well as Z-3-hexenyl acetate at early growth stages observed here may not attract female parasitoids but may have an indirect benefit on their survival and fecundity by influencing nectar production in adjacent weed or forb species. The obligate braconid parasitoids of WSS have two generations per year and can be found in wheat fields throughout the growing season, so

although there was no significant effect of growth stage or allele on the concentrations of linalool in durum wheat, the increase observed as plants matured may still have a similar influence on parasitoid fitness.

Nonanal, decanal, and α-terpineol play a role in resistance against fungal infection and have been shown to inhibit germination of conidia of several fungal species (Q. Li et al., 2021; Quintana-Rodriguez et al., 2015; Zhou et al., 2014). Increased nonanal and decanal in these interactions not only has negative effects on the membrane integrity of fungal cells but can also reduce total lipid concentration (Q. Li et al., 2021). In certain concentrations, nonanal and decanal serve as attractants and oviposition stimulants to many pest species, including Oriental fruit moth *Grapholita molesta* (Lepidoptera: Tortricidae), Grape berry moth *Paralobesia viteana* (Clemens) and Oriental tobacco budworm *Helicoverpa assulta* (Guenée) (Lepidoptera: Noctuidae) (Cha et al., 2011; C. Wang et al., 2020; Xiang et al., 2017). Spring wheat lines emitted less nonanal and decanal as they matured, while concentrations of these compounds in durum wheat increased with maturity. These compounds are not only emitted as volatile compounds by plants, but are also produced by WSS adults and are proven to stimulate electrophysiological activity in WSS antennae although the effects of these compounds on specific WSS behaviors has not yet been studied (Cossé et al., 2002).

Photosynthetic traits are often correlated with total volatile emissions, especially under stress conditions with increased stress having greater effects on photosynthetic parameters and concentrations of specific VOCs (Jiang et al., 2016; Kask et al., 2016; Tomescu et al., 2017). In studies of plant response in relation to abiotic stressors such as high salinity, heat stress or drought conditions, emission of constitutive terpenes and products of the LOX pathway,

including Z-3-hexenyl acetate, decrease with a decrease in photosynthetic rate (Kask et al., 2016; Tomescu et al., 2017). Increases in transpiration rates and conductance are expected to positively affect volatile emissions since these photosynthesis-related parameters are associated with stomatal opening, providing an easy avenue for volatile compounds to escape the leaf (Escobar-Bravo et al., 2023; Urban et al., 2017). However, volatiles produced in the leaf are able to diffuse through cell membranes, across intercellular spaces and can even cross plant cuticles before being released, which explains why there were slight differences in total volatile emissions between durum and spring wheat despite similarities in photosynthetic parameters such as transpiration rate and stomatal conductance.

While yield is notoriously difficult to predict, increased photosynthetic capacity, low canopy temperatures, and increased stomatal conductance have been shown to be positively correlated with yield and biomass, especially when measured at the grain filling stage (Blum, 1990; Fischer et al., 1998; Furbank et al., 2013; Reynolds et al., 2000). While our measurements were collected prior to the start of grain fill and no significant differences in conductance were observed in either species, increased photosynthetic rate in spring wheat lines with the *3BLc* allele and durum wheat at Zadoks 49 indicates the potential for higher yield in these lines. Changes in the abundance of proteins involved in photosynthesis and reaction to light occur in response to WSS infestation in spring, winter and durum wheat. In a proteomics WSS infestation study using Conan, several proteins associated with photosystem I and photosystem II were downregulated in infested stem tissue, so the increased photosynthetic rate observed in spring wheat lines with the Conan allele *3BLc* may provide these plants with an advantage compared to cultivars with lower photosynthetic rates prior to infestation (Lavergne et al., 2020).

Conclusion

The increased reflectance of susceptible spring wheat leaves in the visible range suggests that WSS and their braconid parasitoids may utilize visual cues while searching for spring wheat host plants, while host seeking behaviors in durum wheat may rely more on leaf or canopy thermal signatures as evidenced by higher reflectance in the NIR regions. However, the visible spectrum between 300 and 700 nm did define either the first or second principal components for both spring and durum wheat, so WSS females are likely influenced by a combination of visual stimuli as well as interactions of these with canopy temperature. Despite the effect on leaves due to canopy temperatures, greater reflectance in the NIR region at early growth stages in both wheat species did not appear to be related to increases in overall volatile emissions or increased transpiration rate. Since these physiological responses are not observed, it seems likely that pathways of volatile production are changing with plant maturity. However, increased photosynthetic rates in early solid spring wheat lines do indicate higher yield potential in early solid spring wheat lines which may help limit yield loss when plants are infested.

Nonanal, decanal, linalool and Z-3-hexenyl acetate can attract both pests and their natural enemies so it is difficult to characterize the effects of these compounds in the tritrophic interaction between wheat, WSS and their endemic parasitoids, however changes in these compounds are associated with cell wall changes which may have effects on larval feeding and subsequent survival. Durum wheat showed an increase in Z-3-hexenyl acetate emissions in susceptible lines, which may also be associated with priming of adjacent wheat plants or even increased production of extrafloral nectaries in nearby weed species that provide benefits to parasitoid populations (Kost & Heil, 2006, 2008). Although the 3B locus in spring wheat does

not appear to be significantly associated with emission of Z-3-hexenyl acetate, amounts of linalool differed between growth stages in spring wheat, which can also affect extrafloral nectar production (Kost & Heil, 2006, 2008). These possible associations have implications for agricultural practices, as the effects of cropping systems can alter VOC emissions through multiple growing seasons (Malone et al., 2022).

Overall, the early stem solidness phenotype does not have the same effects on photosynthetic parameters, volatile emissions or reflectance in spring and durum wheat in the context of how WSS resistance is manifested. Further studies on the associations of reflectance, photosynthesis and secondary metabolism with early stem solidness and continued exploration of the effects of reflectance as well as the effects of the composition of volatile profiles on specific behaviors of WSS and their parasitoids will help further elucidate the mechanisms of host-seeking behavior and host plant resistance in this tritrophic system.

Figure 4.1 Spectral reflectance of spring wheat near isogenic lines containing *3BLa* and *3BLc* alleles at Zadoks growth stages 32 and 49

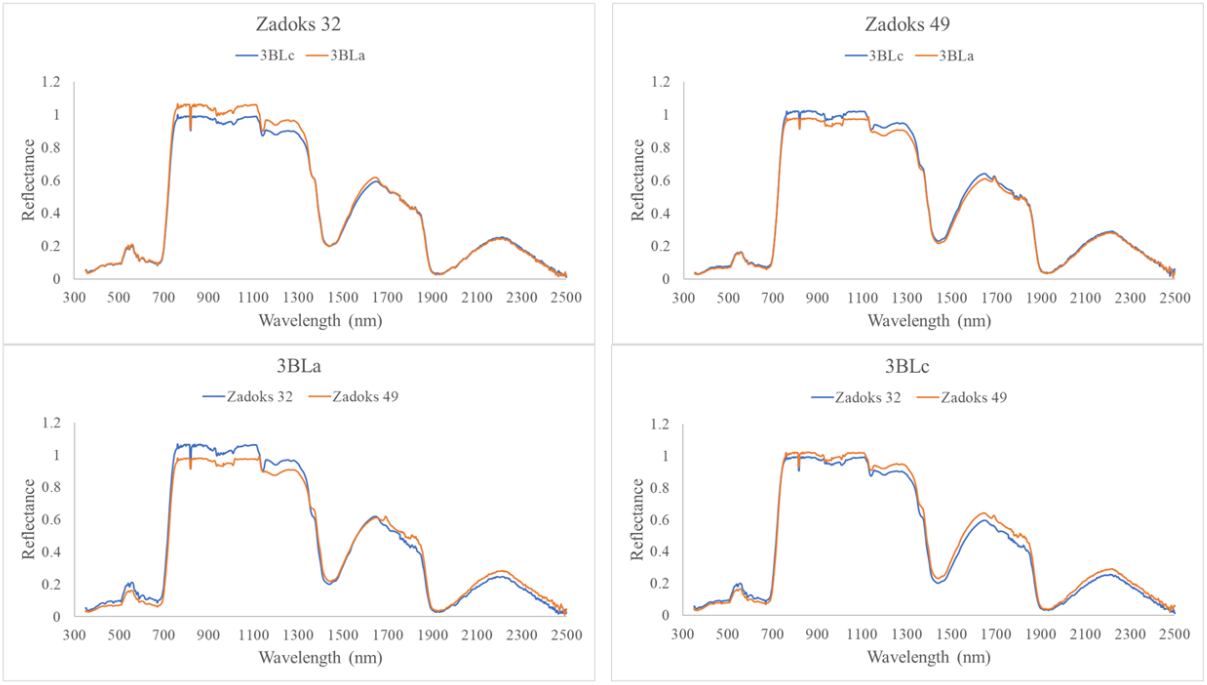

Figure 4.2 Principal component analysis of spring wheat near isogenic lines containing *3BLa* and *3BLc* alleles at Zadoks growth stages 32 and 49

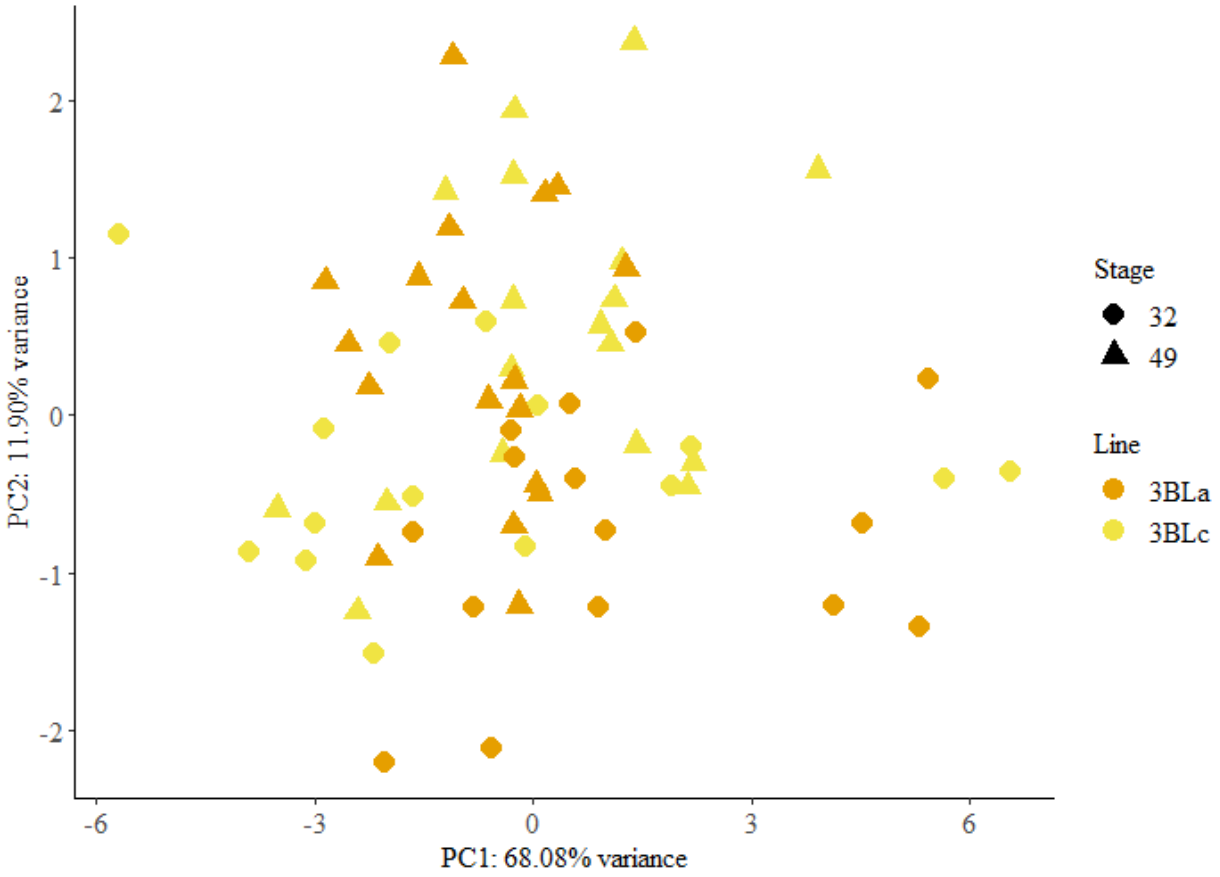

Figure 4.3 Spectral reflectance of durum wheat near isogenic lines containing *3ALa* and *3ALb* alleles at Zadoks growth stages 32 and 49

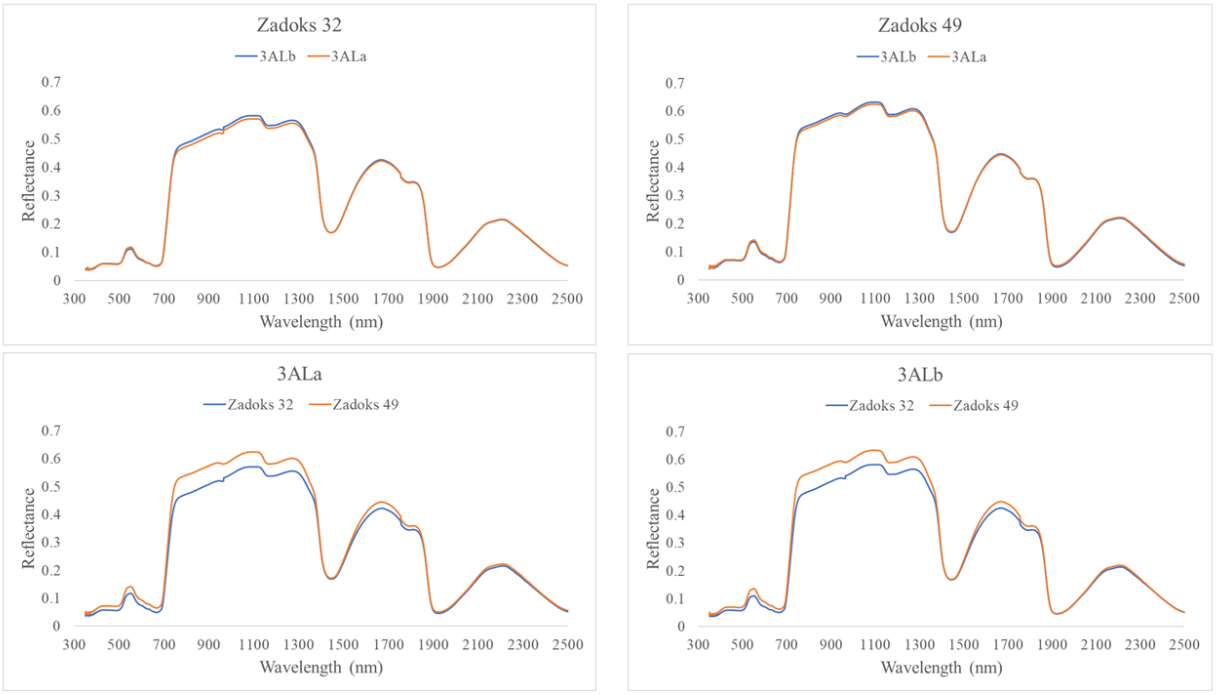

Figure 4.4. Principal component analysis of durum wheat near isogenic lines containing *3ALa* and *3ALb* alleles at Zadoks growth stages 32 and 49

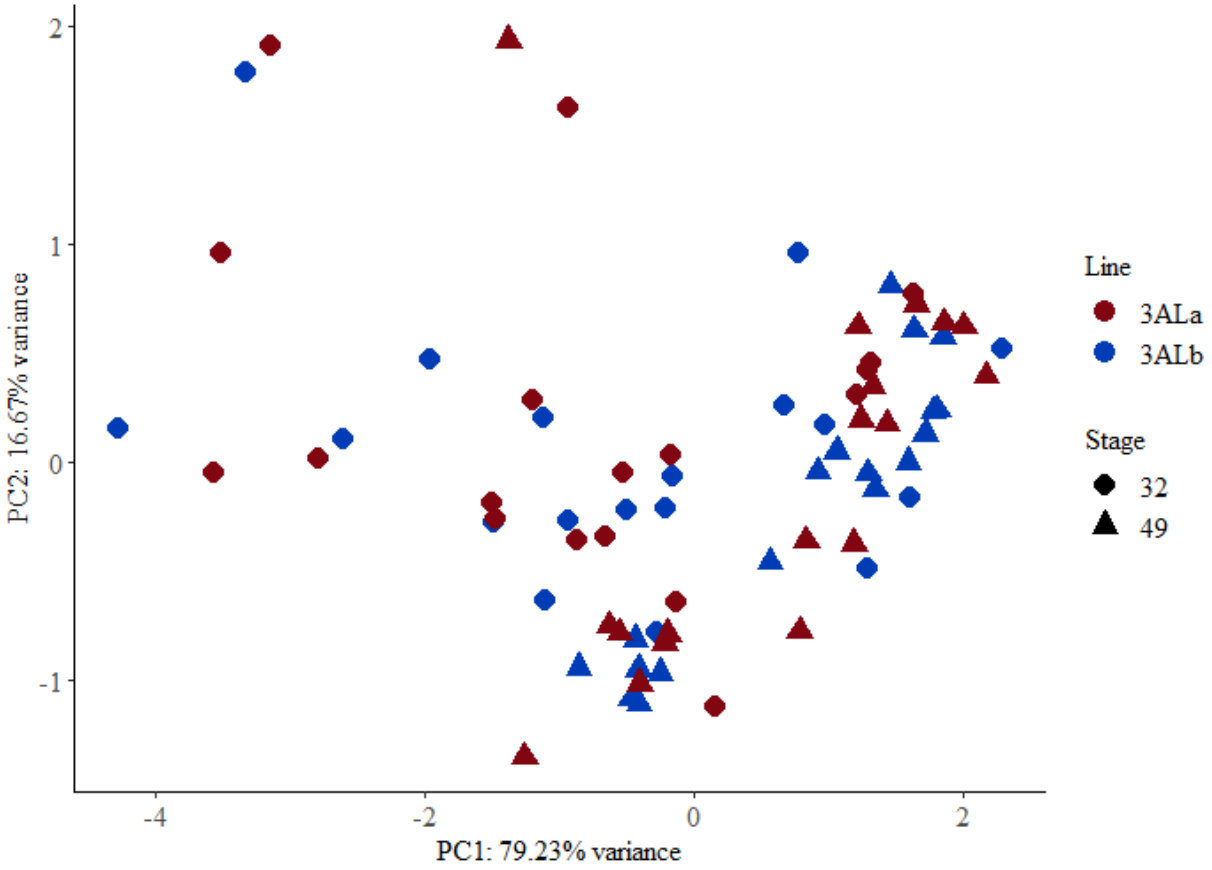

Figure 4.5 Measurements of photosynthetic parameters in near isogenic lines of spring wheat

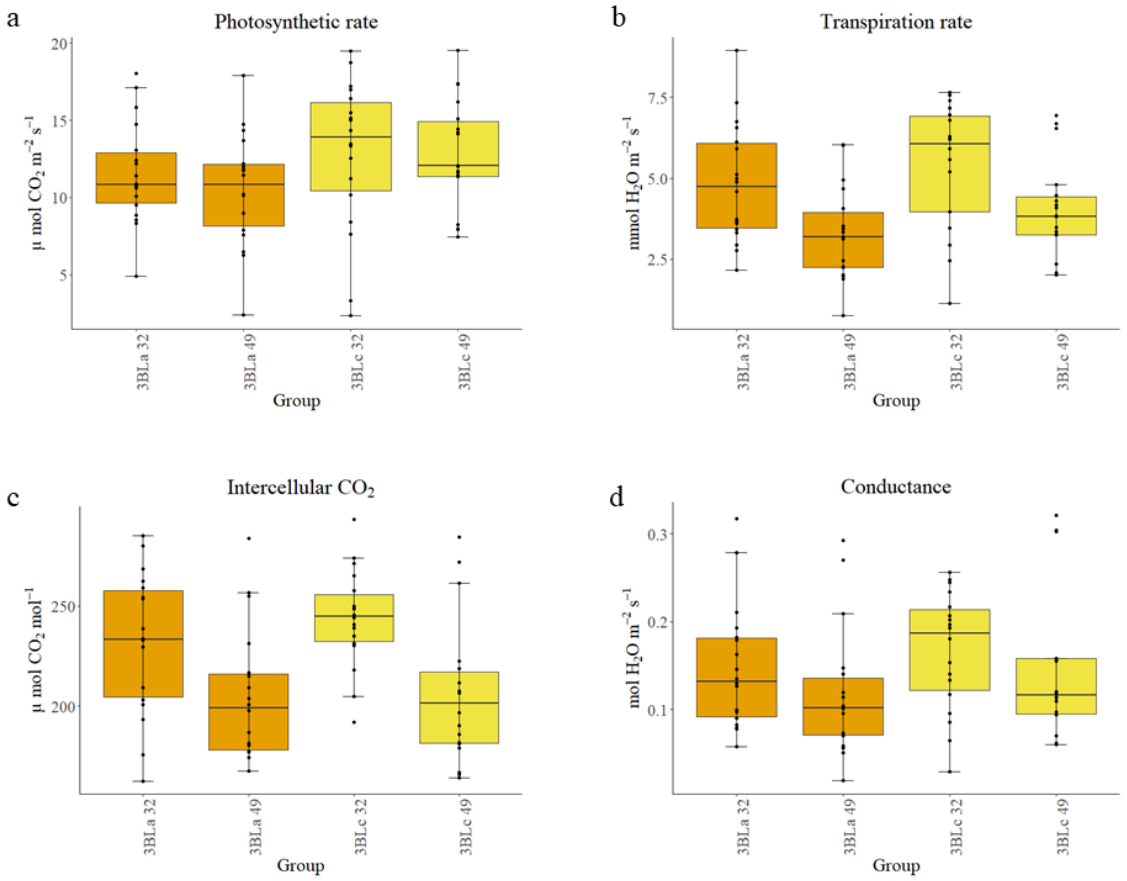

Figure 4.6 Measurements of photosynthetic parameters in near isogenic lines of durum wheat

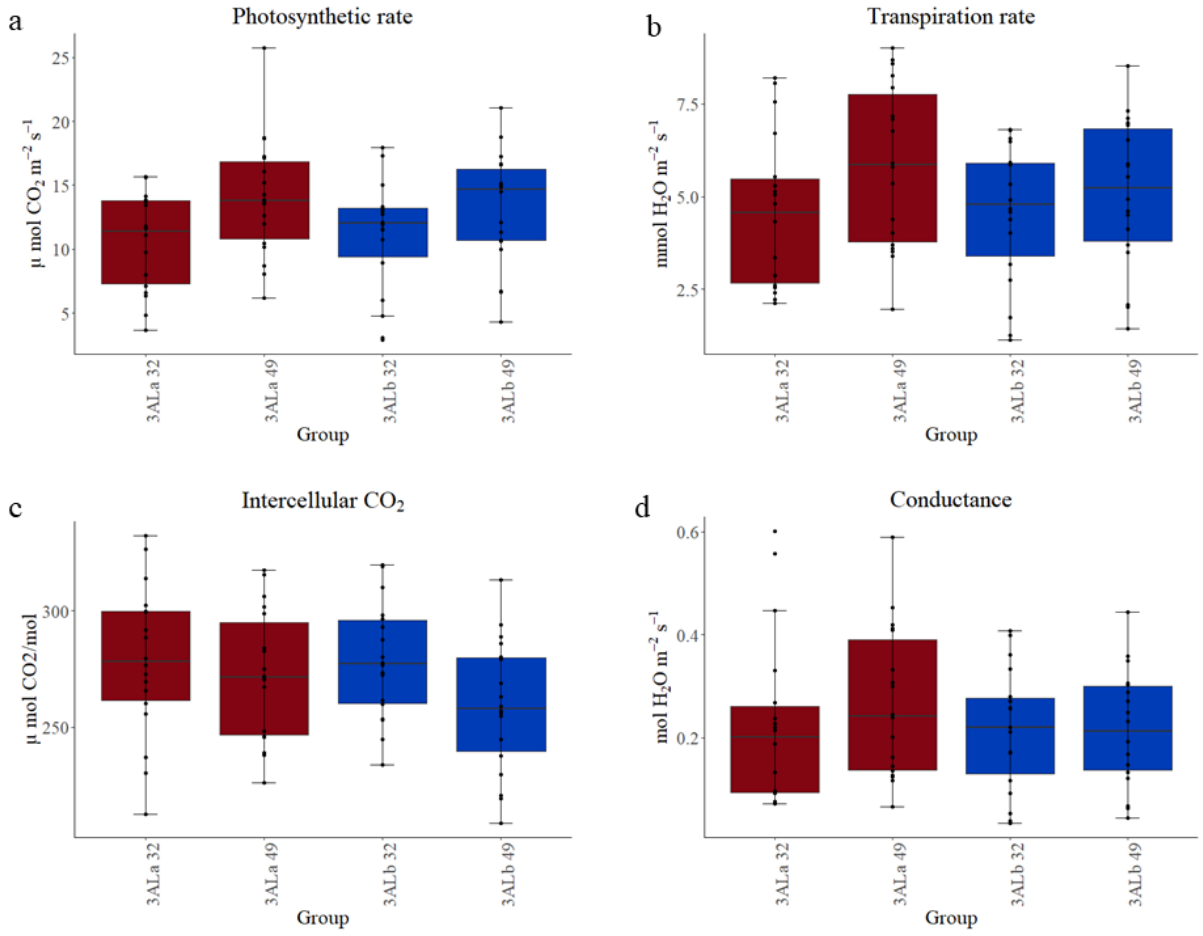

Figure 4.7 Concentrations of volatile organic compounds in near isogenic lines of spring wheat

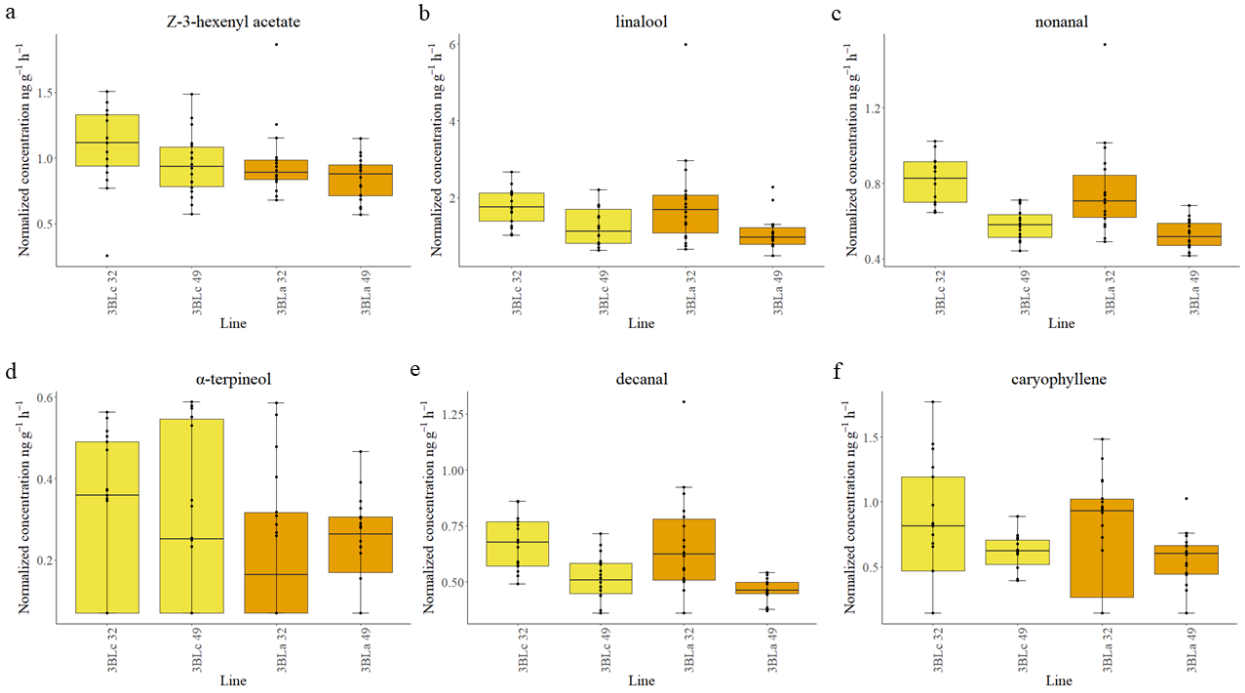

Figure 4.8 Concentrations of volatile organic compounds in near isogenic lines of durum wheat

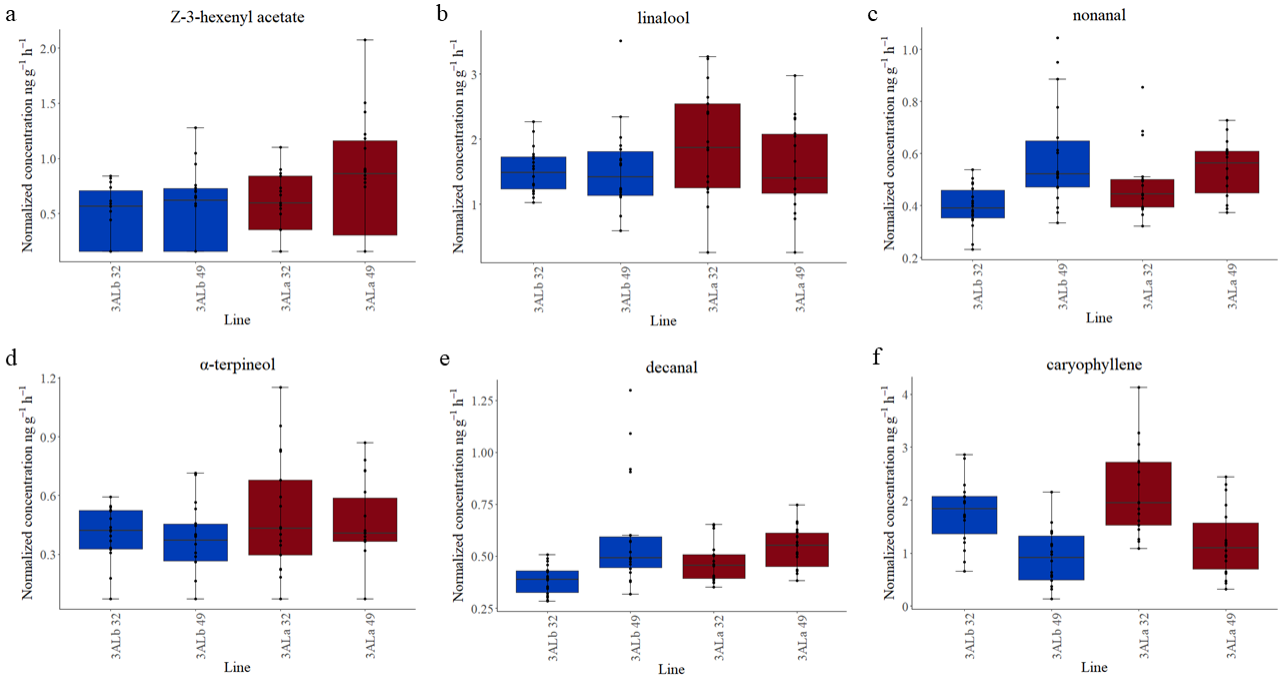

Table 4.1 Primary physiology - MANOVA results from spring wheat near isogenic lines

	Df	Pillai	F value	p-value
Allele	1	.113	1.814	.1386
Stage	1	.799	56.683	<0.05
Temporal Rep	4	1.278	7.046	<0.05
Allele*Stage	1	0.037	.559	.6930
Allele*Temporal Rep	4	.196	.772	.7167

Table 4.2 Primary physiology - One-way ANOVA results from spring wheat near isogenic lines

		Df	F value	p-value
Photosynthetic rate	Allele	1	.0437	.8350
	Stage	1	9.478	<0.05
	Temporal replicate	4	6.469	<0.05
	Allele*Stage	1	.5300	.4694
	Allele*Temporal replicate	4	.7968	.5319
Conductance	Allele	1	1.051	.3094
	Stage	1	0.6107	.4376
	Temporal replicate	4	7.902	<0.05
	Allele*Stage	1	.5821	.4485
	Allele*Temporal replicate	4	.6478	.6307
Intercellular CO2	Allele	1	1.427	.2370
	Stage	1	4.916	.0304
	Temporal replicate	4	7.484	<0.05
	Allele*Stage	1	1.357	.3498
	Allele*Temporal replicate	4	0.7708	.6008
Transpiration rate	Allele	1	.8590	.3578
	Stage	1	5.189	.0263
	Temporal replicate	4	8.187	<0.05
	Allele*Stage	1	.888	.3498
	Allele*Temporal replicate	4	.6914	.6007

Table 4.3 Primary physiology - MANOVA results from durum wheat near isogenic lines

	Df	**Pillai**	**F value**	**p-value**
Allele	1	.0365	.5405	.7065
Stage	1	.5512	17.502	<0.05
Temporal Rep	4	.6100	2.699	<0.05
Allele*Stage	1	.0303	.4450	.7756
Allele*Temporal Rep	4	.1835	.7211	.7715

Table 4.4 Primary physiology - One-way ANOVA results from durum wheat near isogenic lines

		Df	**F value**	**p-value**
Photosynthetic rate	Allele	1	5.2074	.0260
	Stage	1	.5071	.4792
	Temporal replicate	4	7.0993	<0.05
	Allele*Stage	1	.4101	.5244
	Allele*Temporal replicate	4	1.1425	.34532
Conductance	Allele	1	2.478	.1207
	Stage	1	3.298	.0744
	Temporal replicate	4	7.667	<0.05
	Allele*Stage	1	.0578	.8108
	Allele*Temporal replicate	4	.8684	.4882
Intercellular CO2	Allele	1	1.030	.3142
	Stage	1	26.325	<0.05
	Temporal replicate	4	9.245	<0.05
	Allele*Stage	1	.7020	.4055
	Allele*Temporal replicate	4	.2992	.8773
Transpiration rate	Allele	1	3.404	.0699
	Stage	1	19.128	<0.05
	Temporal replicate	4	7.644	<0.05
	Allele*Stage	1	.0364	.8493
	Allele*Temporal replicate	4	1.176	.3306

Table 4.5 Volatile organic compounds – MANOVA results from spring wheat near isogenic lines

	Df	Pillai	F value	p-value
Allele	1	.1612	1.565	.1645
Stage	1	.4715	7.265	<0.001
Temporal Rep	1	.1070	.9759	.4577
Allele*Stage	1	.0985	.8895	.5208
Allele*Temporal Rep	1	.1285	1.201	.3171
Stage*Temporal Rep	1	.0921	.8264	.5697
Allele*Stage*Temporal Rep	1	.0705	.6185	.7384
Residuals	63			

Table 4.6 Volatile organic compounds – one-way ANOVA results from spring wheat near isogenic lines

		Df	F value	p-value
Z-3-hexenyl acetate	Allele	1	3.688	.059
	Stage	1	4.378	.040
	Temporal replicate	1	.0049	.944
	Allele*Stage	1	.0386	.844
	Allele*Temporal replicate	1	.0349	.852
	Stage*Temporal replicate	1	.0581	.810
	Allele*Stage*Temporal replicate	1	.1486	.701
	Residuals	63		
linalool	Allele	1	.0610	.8058
	Stage	1	14.098	.00038
	Temporal replicate	1	.0319	.8588
	Allele*Stage	1	.6271	.4314
	Allele*Temporal replicate	1	1.859	.1775
	Stage*Temporal replicate	1	.0638	.8013
	Allele*Stage*Temporal replicate	1	.1397	.7098
	Residuals	63		
nonanal	Allele	1	1.842	.1795
	Stage	1	43.371	<0.001
	Temporal replicate	1	.0799	.7784
	Allele*Stage	1	.0002	.9879

Table 4.6 continued

	Allele*Temporal replicate	1	.5079	.4787
	Stage*Temporal replicate	1	.0046	.9463
	Allele*Stage*Temporal replicate	1	.1016	.7510
	Residuals	63		
α-terpineol	Allele	1	2.5342	.1164
	Stage	1	.0012	.9722
	Temporal replicate	1	3.1229	.0820
	Allele*Stage	1	.0720	.7893
	Allele*Temporal replicate	1	1.0678	.3054
	Stage*Temporal replicate	1	.9000	.3464
	Allele*Stage*Temporal replicate	1	.1459	.7037
	Residuals	63		
decanal	Allele	1	.5249	.4714
	Stage	1	27.564	<0.001
	Temporal replicate	1	.1526	.6974
	Allele*Stage	1	.5980	.4422
	Allele*Temporal replicate	1	.0720	.7893
	Stage*Temporal replicate	1	.0017	.9670
	Allele*Stage*Temporal replicate	1	.0829	.7744
	Residuals	63		
caryophyllene	Allele	1	.1795	.6732
	Stage	1	5.569	.0214
	Temporal replicate	1	.9455	.3346
	Allele*Stage	1	.0003	.9854
	Allele*Temporal replicate	1	.1634	.6874
	Stage*Temporal replicate	1	1.523	.2218
	Allele*Stage*Temporal replicate	1	.3236	.5715
	Residuals	63		

Table 4.7 Volatile organic compounds – MANOVA results from durum wheat near isogenic lines

	Df	Pillai	F value	p-value
Allele	1	.1362	1.524	.1865
Stage	1	.4847	9.093	<0.001
Temporal Rep	1	.1098	1.192	.3235
Allele*Stage	1	.1488	1.690	.1397
Allele*Temporal Rep	1	.1046	1.129	.3572
Stage*Temporal Rep	1	.1455	1.646	.1511
Allele*Stage*Temporal Rep	1	.0379	.3817	.8878
Residuals	63			

Table 4.8 Volatile organic compounds – one-way ANOVA results from durum wheat near isogenic lines

		Df	F value	p-value
Z-3-hexenyl acetate	Allele	1	4.749	.0331
	Stage	1	2.931	.0918
	Temporal replicate	1	.1944	.6608
	Allele*Stage	1	1.322	.2546
	Allele*Temporal replicate	1	1.155	.2867
	Stage*Temporal replicate	1	.9557	.3320
	Allele*Stage*Temporal replicate	1	.0007	.9787
	Residuals	63		
linalool	Allele	1	1.976	.1647
	Stage	1	.9328	.3378
	Temporal replicate	1	.4011	.5288
	Allele*Stage	1	1.379	.2448
	Allele*Temporal replicate	1	.0017	.9670
	Stage*Temporal replicate	1	1.142	.2892
	Allele*Stage*Temporal replicate	1	.3235	.5715
	Residuals	63		
nonanal	Allele	1	.5232	.4722
	Stage	1	14.852	.0002
	Temporal replicate	1	4.440	.0391

Table 4.8 continued

	Allele*Stage	1	4.835	.0316
	Allele*Temporal replicate	1	.6251	.4321
	Stage*Temporal replicate	1	.4161	.5212
	Allele*Stage*Temporal replicate	1	1.011	.3184
	Residuals	63		
α-terpineol	Allele	1	4.240	.0436
	Stage	1	.7310	.3958
	Temporal replicate	1	4.651	.0349
	Allele*Stage	1	.0602	.8069
	Allele*Temporal replicate	1	4.547	.0369
	Stage*Temporal replicate	1	5.445	.0228
	Allele*Stage*Temporal replicate	1	.3180	.5748
	Residuals	63		
decanal	Allele	1	.1745	.6776
	Stage	1	15.51	.0002
	Temporal replicate	1	2.165	.1461
	Allele*Stage	1	3.852	.0541
	Allele*Temporal replicate	1	.0278	.8682
	Stage*Temporal replicate	1	.0990	.7541
	Allele*Stage*Temporal replicate	1	1.822	.1819
	Residuals	63		
caryophyllene	Allele	1	4.360	.0408
	Stage	1	35.116	<0.001
	Temporal replicate	1	.1411	.7085
	Allele*Stage	1	.0693	.7932
	Allele*Temporal replicate	1	2.055	.1567
	Stage*Temporal replicate	1	6.986	.0103
	Allele*Stage*Temporal replicate	1	.0000	.9947
	Residuals	63		

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CHAPTER FIVE

CONCLUSION

Resistance to WSS conferred by the solid stem phenotype has been the focus of breeding efforts since the discovery of the solid stem Rescue allele, S-615, and the planting of cultivars with solid stems remains a foundational approach among producers in areas with high WSS pressure. However, WSS can still cause considerable stem cutting and yield losses in fields planted with solid stemmed cultivars since the expression of the solid stem phenotype varies significantly depending on planting density and environmental conditions such as light, water and nutrient availability (Beres et al., 2011; Beres et al., 2017; Delaney et al., 2010; Pluta et al., 2021; Roemhild, 1954). Additionally, cultivars with solid stems appear to have negative impacts on the populations of endemic braconid parasitoids of WSS, *B. cephi* and *B. lissogaster* (Rand et al., 2020; Rand et al., 2012; Runyon, 2001; Wu et al., 2013). It is therefore crucial to explore additional sources of resistance to WSS outside of the solid stem phenotype. Here, we used untargeted metabolomics to explore the mechanisms of host plant resistance in oat, a combined untargeted metabolomic and transcriptomic approach to identify key differences associated with early stem solidness and plant development in spring and durum wheat, and an analysis of primary physiological and secondary metabolic characteristics to identify mechanisms related to plant development and the early solidness phenotype in spring and durum wheat.

Metabolites involved in the phenylpropanoid pathway, phospholipid biosynthesis and signaling, cellular organization and tissue structure and plant defense were differentially expressed between oat and wheat, suggesting potential mechanisms involved in the resistance of oat to WSS infestation. Compounds tentatively identified as oleate, 5-hydroxyconiferyl alcohol,

and 16-feruloyloxypalmitate were found in higher abundance in oat, indicating that there may be effects on suberin and lignin biosynthesis and the ratio of lignin subunits in stem tissue. Effects on cell elongation and stem growth were also suggested by differential expression of several IAA-conjugates between oat and wheat. Downregulation of genes and proteins involved in lignin biosynthesis has been observed in WSS infestation studies using spring wheat as well as winter wheat, indicating the importance of cell wall structure and stem tissue composition in resistance to WSS (Biyiklioglu et al., 2018; Lavergne et al., 2020). Metabolites related to plant defense were also upregulated in oat, including salicylic acid-related compound salicyl-6-hydroxy-2-cyclohexene-on-oyl and benzoxazinoid DIBOA-β-D-glucoside. Oat stem tissue also had higher abundance of several phospho- and galacto-lipids compared to wheat. In studies of plants infested by WSS, additional compounds involved in benzoxazinoid and lipid metabolism were implicated in the response to larval feeding in both spring and winter wheat (Biyiklioglu et al., 2018; Lavergne et al., 2020). The metabolic differences between oat and wheat have been observed in other plant hosts of WSS and are indicative of effects on the structure and composition of stem tissue which may in turn have effects on larval feeding and subsequent development.

Comparison of the transcriptome and metabolome of hollow stemmed lines and lines with an early stem solidness allele in spring and durum wheat revealed differences in transcript and metabolite abundance with potential effects on total lignin content, lignin subunit proportion and production of semiochemicals, including those which are detected by WSS when searching for host plants. Differences in carbohydrate and lipid abundance were also observed in both spring and durum wheat, and while lipids abundance varied by growth stage or allele in both

species, increased carbohydrate abundance was observed in spring and durum wheat cultivars with the early solidness allele. Lipid changes are induced with infestation of spring and winter wheat by WSS (Biyiklioglu et al., 2018; Lavergne et al., 2020). Stem architecture, lignin subunit composition, protein: carbohydrate ratio, and the lipidome have the potential to affect host plant selection by WSS and larval fitness. Additionally, *TaVPE3cB*, *TdDof* and *TaDof,* genes associated with stem solidness, programmed cell death, and cell wall development were also identified in the spring and durum wheat datasets (Cheng et al., 2019; Liu et al., 2023; Nilsen et al., 2020). Increased expression of *TaVPE3cB* was observed in Reeder and *3BLa* lines, a result observed in previous studies of hollow stemmed cultivars and associated with thickening of cell walls and increased rate of programmed cell death (Cheng et al., 2019; Liu et al., 2023). These results suggest that *TaVPE3cB* may be responsible for the hollow stemmed phenotype observed in Reeder and NILs containing the *3BLa* allele. *TdDof* in durum wheat and its ortholog in spring wheat *TaDof,* candidate genes for *SSt1* were not differentially expressed between hollow stemmed lines and lines with early stem solid alleles in either spring or durum wheat.

Early stem solidness alleles were not found to be associated with the similar patterns of spectral reflectance, photosynthetic parameters or volatile compound emission in spring and durum wheat. Spectral reflectance of flag leaves at early and late growth stages revealed differences between hollow and early solid spring wheat lines in the visible and near-infrared (NIR) range, while durum wheat showed the greatest differences between growth stages in the NIR range. These results support the idea that WSS, as well as associated braconid parasitoids, likely use visual cues as well as variations in leaf and canopy temperature to locate suitable hosts. Higher reflectance and photosynthetic rate were also observed in Conan and are likely

related to the increased moisture content in Conan leaves and stems at early growth stages that was observed in previous studies (Varella et al., 2016; Varella et al., 2017). The plant volatile compound Z-3-hexenyl acetate was emitted in greater concentrations from susceptible durum lines, while linalool differed in concentration between growth stages in spring wheat lines. These compounds can prime nearby wheat plants and induce cell wall changes or attract parasitoids in addition to attracting WSS (Ameye et al., 2015; Ameye et al., 2020; Kost & Heil, 2006; Pérez, 2009; Piesik et al., 2008; Whitman & Eller, 1992). They also have the capability to stimulate production of nectar from flowers and extrafloral nectaries of other nearby species, which parasitoids can use as a source of supplemental carbohydrates, improving biological control of WSS (Kost & Heil, 2006, 2008).

There are many common themes that connect the experiments outlined here. First, constitutive or induced changes in cell walls or cellular organization, lignin content and composition, tissue structure and tissue moisture were identified in all experiments. In oat, increased abundance of metabolites tentatively identified as 5-hydroxyconiferyl alcohol, and 16-feruloyloxypalmitate were observe, and in spring and durum wheat near isogenic lines transcripts and metabolites associated with the phenylpropanoid pathway appeared to be related to early stem solidness. Our results support the findings from infestation studies in spring and winter wheat which identified genes and proteins involved in lignin biosynthesis which were differentially expressed in response to larval feeding and demonstrate that cell wall structure and the molecular architecture of the stem is important to plant resistance to WSS (Biyiklioglu et al., 2018; Lavergne et al., 2020). Secondly, lipid biosynthesis and signaling pathways were affected by infestation in oat, and also in uninfested durum and spring wheat comparisons using resistant

and susceptible alleles. Previous studies have also implicated the importance of lipid metabolism in the WSS system, including a study of uninfested durum which showed that solid stemmed durum lines had higher lipid content than *pithless* lines and an infestation study using spring and winter wheat which also identified lipid involvement in plant response to WSS infestation (Lavergne et al., 2020; Nilsen et al., 2020). Finally, we found that early stem solidness alleles in durum and spring wheat often had differing effects on primary and secondary metabolism depending on the genetic background. There were several instances where parental lines had a unique response, even in comparison to their near isogenic lines with the same alleles, indicating that other mechanisms may be related to the WSS resistance observed in resistant lines, in addition to the early stem solidness trait. While the solidness trait, including early stem solidness, has been shown to be consistent in different genetic backgrounds, our results indicate that other characteristics such as reflectance, concentration of volatile compounds, metabolite abundance and even expression of gene transcripts are likely affected by regions of the genome outside the 3B and 3A QTLs. These characteristics can have significant effects on the behavior of adult and larval WSS as well as their parasitoids, which has important implications for cultivar development. Future studies should utilize multiple pairs of near isogenic lines to capture the effect of these QTLs on primary metabolism, secondary metabolic profiles and WSS resistance. Breeding programs seeking to develop new cultivars resistant to WSS should also be conscious of the possibility of unintended effects on characteristics related to insect behavior and nutrition in order to support the tritrophic interaction between WSS, parasitoid and plant and maximize biological control efforts.

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