2016

A Molecular Genetic and Physiological Comparison of the Hybrid Necrosis Response in Wheat (Triticum Aestivum L.) to Biotic and Abiotic Stress Responses

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A MOLECULAR GENETIC AND PHYSIOLOGICAL COMPARISON OF THE HYBRID NECROSIS RESPONSE IN WHEAT (*TRITICUM AESTIVUM* L.) TO BIOTIC AND ABIOTIC STRESS RESPONSES

BY

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A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Plant Science

South Dakota State University

2016

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Plant Science degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest appreciation to my advisor, Dr. Karl D. Glover, for providing constant support, encouragement, and supervision. It would not have possible to complete this dissertation and achieve my goal without his guidance and persistent help. I would also like to acknowledge Dr. William A. Berzonsky for providing the opportunity to work with his wheat breeding program and for continuous guidance. I have also greatly benefitted from the steadfast support and intellectual guidance of my committee members Dr. Jose L. Gonzalez-Hernandez and Dr. Shaukat Ali. I am greatly thankful to Dr. Jose L. Gonzalez-Hernandez for his time, guidance and suggestions in RNA-seq project. I would also like to thank for my graduate committee member Dr. David Wiltse for support on this dissertation.

I greatly appreciate the help from Jonathan Kleinjan, Steven Kalsbeck, Julie Thomas, Greg Redenius, and Jesse Cameron for technical support and for the timely management of my greenhouse and lab activities. I would like to thank the USDA-ARS Cereal Crops Research Unit, Fargo, ND, for providing lab resources to screen molecular markers. I appreciate the advice and help provided by Dr. Shiaoman Chao and Mary Osenga.

I would like to extend special thanks to Michaellong Tran for his help in the RNA–seq project. I am greatly thankful to Stacy Dreis for her time on corrections and suggestions on this dissertation. I would also like to acknowledge the support I got from Siddhi Bhusal. I would also like to thank Arjun and Geetanjali for companionship during my graduate program. I would like to extend my thanks to David, Tulsi, Deepak,

Krishna, Sidrat, Bandana, Sudha, Kopila, Debendra, Navjot, Jagdeep, Sai, Thumbiko, and Yaqoob for providing entertainment, camaraderie, and friendship.

I am very grateful to my loving wife, Shanti Kandel. This degree would not have possible without her unwavering support, patience, and inspiration. I would like to extend my thanks to my daughters, Melissa and Shanvi. Their smiles always have been the source of my energy.

Funding for these projects were provided by the South Dakota Wheat Commission.

CONTENTS

ABBREVIATIONS

ABA = Abscisic acid

AMP = adenosine monophosphate,

APX = Ascorbate peroxidase

BA = Brassinosteroids

CAM = Calmodulin

CDPKs = Calmodulin – domain protein kinases

CAPKs = Calcium dependent protein kinases

CRTs= Calreticulins

CWDEs = Cell wall degrading enzymes

DAMP = Damage associated molecular patterns

ETI = Effector triggered immunity

FPKM = Fragments per kilobase of transcript per million mapped reads

GMP = guanosine monophaosphate.

GPX = Glutathione peroxidase

GST = Glutathione –S – transferase

HR = Hypersensitive response

HSP = Heat shock protein

IMP = Inosine monophosphate,

JA = Jasmonic acid

MAMP = Microbe - associated molecular patterns

MAP Kinases = Mitogen activated protein kinases

MKK = MAP kinase kinase

PAMP = Patterns associated molecular patterns

PCA = Principal component analysis

PR-proteins = Pathogenesis related proteins

PRRs = Plant recognition receptors

PTI = Pathogen-associated molecular pattern - triggered immunity

RCLs = Receptor like kinases

RLPs = Receptor like proteins

RPKM = reads per kilobase of transcript per million mapped reads

SA = Salicylic acid

SOD = Superoxide dismutase

SSR = Simple sequence repeat

WAKs = Wall associated protein kinases

XDH = Xanthine dehydrogenase

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ABSTRACT

A MOLECULAR GENETIC AND PHYSIOLOGICAL COMPARISON OF THE HYBRID NECROSIS RESPONSE IN WHEAT (*TRITICUM AESTIVUM* L.) TO BIOTIC AND ABIOTIC STRESS RESPONSES

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Expression of hybrid necrosis in plants can lead to a significant reduction in productivity, or even lethality. Epistatic interactions between divergent loci that have evolved through evolution are proposed as being responsible for the genetic incompatability that is expressed as hybrid necrosis. Hybrid necrosis can also represent an obstacle to the transfer of desirable traits from wild, related species to domesticated bread wheat. In wheat, expression of dominant complementary genes *Ne1* and *Ne2,* located to chromosomes 5B and 2B in a hybrid results the production of necrotic leaf tissue, stunted plant growth, and reduced grain yield, which are similar plant responses to many biotic and abiotic stresses. This study was undertaken to compare the molecular genetic and physiological hybrid necrotic responses in wheat with those typically observed for biotic and abiotic stresses. Objectives were to; (1) measure levels of reactive oxygen species (ROS) and , xanthine dehydrogenase (XDH) during wheat hybrid necrosis, (2) assess the impact of temperature on the expression of hybrid necrosis, (3), assess the allelic interactions responsible for necrosis, and (4) characterize the genes up-regulated during the expression of hybrid necrosis in wheat. The production of H_2O_2 in hybrid necrotic plants was not significantly different than in non-necrotic parents, suggesting that unlike some stress responses, elevated H_2O_2 levels are not responsible for

causing programmed cell death and tissue necrosis in wheat. Reduced plant growth and yield in the allopurinol treated plants, and those plants expressing suppression of XDH affirms the role of XDH in purine catabolism and most likely nitrogen reassimilation in plants. Similar to the temperature sensitive expression of some disease resistance genes in wheat, expression of necrosis symptoms was reduced when hybrids were grown at 30^{O}C. The SSR markers; Xbarc7, Xbarc13 and Xwmc344 are linked to the *Ne2* gene at distances of 3 cM, 4 cM, and 6 cM, respectively, and Xgwm639 is linked at 11 cM with *Ne1*. There was an observed dosage effect associated with the *Ne* alleles, and both 'Alsen' and synthetic hexaploid wheat line 'TA 4152 -37' expressed moderate necrosis due to the Ne^{m} alleles. Gene expression analyses revealed that defense signaling genes were highly up-regulated during the expression of hybrid necrosis along with the activation of several antioxidant enzymes. Detection of high levels of polyamine oxidase activity during hybrid necrosis suggests that this enzyme could be the main source of ROS responsible for tissue cell death. Genes typically encoding for plant recognition receptors (PRRs), pathogenesis–related proteins, antioxidant enzymes, calcium regulation, protein kinases and ethylene biosynthesis were all up-regulated during expression of wheat hybrid necrosis. Results support the concept that hybrid necrotic symptoms in wheat are the byproduct of an autoimmune type of response similar to many responses invoked by wheat plants when exposed to a biotic, or abiotic stress.

CHAPTER 1: Literature Review

Hybrid Necrosis in Plants

Hybrid necrosis in crops is characterized by tissue necrosis, stunted growth, and often lethality (Hermsen, 1963a, 1963b; Bomblies and Weigel, 2007). Hybrid necrosis represents a barrier to gene transfer between cultivars and related species. It is considered a postzygotic hybridization barrier resulting from genetic incompatibility (Bomblies and Weigel, 2007). Along with hybrid necrosis, hybrid sterility, and hybrid inviability are the other postzygotic reproductive barriers that are also the result of genetic incompatibility (Chen et al., 2016).

The Dobzhansky-Muller (DM) model (Dobzhansky and Dobhansky, 1937; Muller, 1942; Bomblies and Weigel, 2007) describes the adaptive evolution process to explain the incompatibility associated with hybrid necrosis. During the process of evolution, and as ancestral lines diverge during the process of speciation, they can exhibit independent substitutions in the genomes. The substitutions have no negative effects on the native genome; however, when the diverged lineages are hybridized, a genetic interaction of the diverged loci (DM loci) results in genetic incompatibility causing necrosis (Bomblies and Weigel, 2007).

In tomato (*Solanum lycopersicum* L.), the cloned gene *Cf-2* confers resistance to *Cladosporium fulvum* and when *Cf-2* was transferred together with its co-evolved *RCR3* loci to domestic tomato, *S. lycopersicum* plants exhibited resistance to *Cladosporium*

without developing necrosis (Krüger et al., 2002). However, when the *Cf-2* gene was transferred into the *RCR3* background of S. *lycopersicum*, tissue necrosis was observed (Krüger et al., 2002; Santangelo et al., 2003). This is an example of what can occur when divergent loci interact and the interacting loci have co-evolved.

Several explanations have been proposed for how in the course of evolution genetic incompatibility is expressed when there is post-zygotic hybridization. Geographical separation during speciation may result in such incompatibilities (Coyne and Orr, 2004). Plants growing in different geographies under different environmental conditions can develop genetic incompatibilities. For example, temperature differences can result in the the suppression of some genes, and the over-expression of others. Low temperatures can promote the expression of deleterious alleles, while high temperatures can suppress immunological responses typified by genetic incompatibilities (Bergelson, 2010; Alcazar and Parker, 2011; Hua, 2013). During evolution, genomic changes can become permanently fixed.

The co-evolution of plant resistance genes with pathogen virulence and avirulence genes during evolution may also have contributed to genetic incompatibilities between plants (Chen et al., 2016), as natural selection based on resistance is a driver of divergence between resistance genes (Jones and Dangl, 2006). This could be why resistance genes in plants are more diverse compared with other gene types (Bomblies, 2009). In Arabidopsis, disease resistance genes are highly polymorphic (Clark et al.,

2007), and epistatic interactions between host pathogen resistance genes has been proposed as a reason for the genetic incompatibility associated with hybrid necrosis (Presgraves, 2010).

Other plant genetic factors possibly contributing to genetic incompatabilities include; transposons, meiotic drive elements, and gamete-killing segregation distorters, which due to their inhibition of gene expression have been designated "selfish" genes (Presgraves, 2010). When suppressed "selfish" genes from one species are transferred to another species during hybridization, the result could be hybrid necrosis. Alternatively, host genes that compensated for the "selfish" genes could result in genetic incompatability when transferred to other species (Presgraves, 2010). These "selfish" genes are prevalent in hybrid sterility (Presgraves, 2010; Rieseberg and Blackman, 2010).

Polyploid speciation is an important evolutionary process in plants (Rieseberg and Willis, 2007). Polyploidization results in the full duplication of a genome after hybridization, which has been implicated in the genetic diversification of plants (Soltis et al., 2007). The loss of full genomic sequences was reported in the F_1 and allotetraploids between *Ae. longissimi* x *Ae. umbellulata* and *Ae. Sharonensis* x *Triticum monococcum (*Shaked et al., 2001). In Triticale, a high level of gene elimination was observed immediately after hybridization, or after chromosome doubling (Ma and Gustafson, 2008). More changes were observed within the rye genome than that of the wheat genome, resulting in asymmetry between the species genomes (Ma and Gustafson, 2008).

Newly formed polyploids also often exhibit abnormal recombination during meiosis (Levin, 1975). Variation in ploidy number has been observed in many species (Soltis et al., 2007), which is a driver of significant differences among plant lineages (Soltis et al., 2007; Rieseberg and Willis, 2007).

Hybrid Necrosis in Wheat

Hybrid necrosis in wheat is represented by a gradual death of leaf or leaf sheath tissue after specific hybrid combinations are produced (Hermsen, 1963a). Hybrid necrosis can be lethal, or cause severe stunting in wheat, and it is the result of the interaction of two complimentary dominant genes (*Ne1* and *Ne2*) (Tsunewaki, 1960). The *Ne1* and *Ne2* genes are located on chromosomes 5B and 2B, respectively (Tsunewaki, 1960; Zeven, 1972; Nishikawa et al., 1974), and the necrosis they induce in intraspecific wheat hybrids is called type I necrosis (Tsunewaki, 1960, 1970; Chu et al., 2006; Mizuno et al., 2010).

Common bread wheat is an allohexaploid (2n = 6x = 42, AABBDD genomes). It evolved as a result of a natural cross between the tetraploid wheat (*Triticum turgidum* L., 2n = 4x = 28 AABB genome) and the diploid wild wheat, *Aegilops tauschii* Coss (2n = 2x = 14, DD genome; Kihara, 1944; McFadden and Sears, 1944). Synthetic hexaploid wheats can be produced by artificially hybridizing a tetraploid wheat with *Ae. tauschii*, followed by the production of an amphiploid from the triploid F_1 hybrid (McFadden and Sears, 1944; Kihara and Lilienfeld, 1949; Matsuoka and Nasuda, 2004). Species

possessing multiple genomes exhibit a higher frequency of genetic incompatibility than diploid species (Hatano et al., 2012), and the mechanism of necrosis differs between intraspecific and interspecific hybrids. In interspecific hybrids, the interaction is called either a type II, or type III necrosis, resulting from an epistatic interaction between the A, B and D genomes (Mizuno et al., 2010). Both genes that induce necrosis in intraspecific wheat hybrids are derived from the B genome.

Characterization and Mapping of Ne1 and Ne2 genes

Although hybrid necrosis in wheat is caused by the complementary interaction of the *Ne1* and *Ne2* genes, the degree of severity can differ depending on the allele combinations resulting from the cross (Hermsen, 1966, Hermsen, 1963a, 1963b; 1966). Multiple alleles for *Ne1* and *Ne2* and their intereaction are reportedly responsible for different levels of necrosis. For example, three alleles at the *Ne1* locus that induce weak ($Ne1^{w}$), moderate ($Ne1^{m}$), or strong ($Ne1^{s}$) necrosis have been documented (Hermsen, 1963; Zeven, 1972; Singh et al., 1992). While, five alleles at the *Ne2* locus induce weak ($Ne2^{w}$), moderate to weak ($Ne2^{mw}$), moderate ($Ne2^{m}$), moderate to strong ($Ne2^{ms}$) and strong ($Ne2^{s}$) necrosis (Hermsen, 1963; Zeven, 1972; Singh et al., 1992). Thus, different allelic combinations of *Ne1* and *Ne2* genes determine the degree of necrosis exhibited in F_1 hybrids (Hermsen 1963a; Singh et al., 1992; Takumi et al., 2013).

In addition to the weak, moderate, or strongly necrotic allelic combinations, necrosis is also affected by gene dosage (Hermsen 1963b; Zeven, 1972). When genotypes with weak necrosis alleles (*Ne1*^{w}) were hybridized with genotypes possessing the strong necrosis *Ne2* allele (*Ne2*^{s}), a higher level of necrosis was observed (Takumi et al., 2013). Various combinations of necrosis-inducing alleles can cause plants to die early, or result in signficantly reduced yields, or conversely, have little, or no impact on plant performance (Hermsen, 1963a; Takumi et al., 2013).

Using telosomics, Nishikawa et al. (1974) were the first to map the wheat hybrid necrosis genes, with the *Ne1* and *Ne2* genes mapping to the short arm of chromosome 2B (10.5 ± 2.0 cM from the centromere), and the long arm of chromosome 5B (9.4 ± 1.5 cM from the centromere), respectively. Chu et al. (2006) later used simple sequence repeat (SSR) markers to identify several closely linked markers to both *Ne1* and *Ne2*.

Geographic Distribution of Ne1 and Ne2 Genes

The *Ne1* and *Ne2* genes are widely distributed among wheat cultivars and wild species (Tsunewaki, 1992; Pukhalskiy et al., 2000; Vikas et al., 2013). Though frequencies differ, both genes are distributed throughout the world (Pukhalskiy et al., 2000). Approximately, 55.1% of wheat genotypes from North America and 6.7% of wheat from Africa carry *Ne2*; whereas, 7.5% of wheat from North America and 47.9% of wheat from Africa possess *Ne1* (Pukhalskiy et al., 2000). Thus, there are more genotypes

with *Ne2* in North America, and more with *Ne1* in Africa. Of 104 Indian bread wheat varieties tested, 65.4% possessed the *Ne2* gene and 7.7% had the *Ne1* gene (Vikas et al., 2013). Within Australian wheats; *Ne1* and *Ne2* were present at frequencies of 25.4% and 18.8%, respectively (Pukhalskiy et al., 2000).

Breeding efforts designed to enhance disease resistance have increased the frequency of the *Ne2* gene. This is because *Ne2* is tightly linked with the leaf rust (*Puccinia triticina* Erikss.) resistance gene *Lr13* (Singh, 1993). Recent work by Zhang et al. (2016) suggests that *Lr13* could represent the $Ne2^m$ gene. Also, *Lr13* gene is tightly linked with *Lr23*, another leaf rust resistance gene. Both the *Lr13* and *Lr23* genes have been extensively employed in wheat breeding programs. The frequency of *Ne2*, may also be influenced by efforts to transfer other widely utilized resistance genes to wheat, such as the stem rust resistance genes; *Sr19, Sr23, Sr36,* and *Sr40*, as well as the stripe rust resistance genes; *Yr31* and *Yr32* (McIntosh et al., 1995), which like *Ne2* are located to chromosome 2B. Indications are that selection for rust resistance has increased the frequency of *Ne2* in North American germplasm (Bomblies and Weigel, 2007; Vikas et al., 2013).

Reactive Oxygen Species (ROS) Generation in Plants

Superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) are three major forms of reactive oxygen species (ROS) generated in plants (Apel and Hirt, 2004). During metabolic processes such as photosynthesis and respiration, plants produce ROS in chloroplasts, mitochondria, peroxisomes, and other organelles within the cell (Tripathy and Oelmüller, 2012). Plants activate enzymes which are responsible for the production of ROS. For example nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidases are responsible for the production of superoxide. Other classes of oxidases, such as oxalate oxidase and amine oxidases have can also generate H_2O_2 (Hu et al., 2003; Mittler et al., 2004). Other enzymes, such as alternate oxidases and glycolate oxidases have also been implicated in production of ROS (Apel and Hirt, 2004; Mittler et al., 2004). Xanthine dehydrogenase has been associated with the generation of ROS during several host–rust interactions (Montalbini, 1992a, 1992b, 1995).

Under normal physiological conditions, ROS are scavenged by antioxidant enzymes, and some of the major antioxidant enzymes in plants are; superoxide dismutase, catalase, ascorbate peroxidase, and glutathione peroxidase (Apel and Hirt, 2004; Tripathy and Oelmuller, 2012). Superoxide dismutase is often involved in the initial plant response to a stress as it catalyzes the conversion of superoxide to hydrogen peroxide (H_2O_2). Detoxification of H_2O_2 is subsequently performed by catalase, ascorbate peroxidase, and glutathione peroxidase (Apel and Hirt, 2004). An overproduction of ROS in excess of

what can be scavenged by antioxidants leads to cell death (Apel and Hirt, 2004), and the equilibrium between the production of ROS and what can be scavenged is affected by biotic and abiotic stresses (Malan et al., 1990, Prasad et al., 1994; Apel and Hirt, 2004).

Reactive oxygen species are a key component to plant defense mechanisms in response to insects and pathogens (Heath, 2000; Kenton et al., 2008; Liu et al., 2010). Upon exposure to an insect or pathogen, plant enzymes responsible for the generation of ROS are activated. Consequently, some H_2O_2 is produced directly (Yoda et al., 2003; 2006), and superoxide is converted to H_2O_2 (Apel and Hirt, 2004). Hydrogen peroxide diffuses through the cell more rapidly than superoxide, and acting as a signaling molecule, it triggers programmed cell death in the plant (Dangl and Jones, 2001; Coll et al., 2011).

ROS Generation during Hybrid Necrosis

Higher generation of superoxide and H_2O_2 has been observed in association with the expression of necrosis in hybrid wheat (Khanna-Chopra et al., 1998; Sharma et al., 2003). With the progressive development of necrotic tissue, ROS generation also increases (Khanna-Chopra, Dalal, et al., 1998, Sharma et al., 2003), and Dalal and Khanna-Chopra (2001) showed an increase in the activity of the antioxidants, ascorbate peroxidase and glutathione peroxidase. In the same study, there was lower catalase activity as symptoms of necrosis developed, and the production of ROS in excess of

antioxidants was likely a cause of tissue cell death (Dalal and Khanna-Chopra, 2001; Sharma et al., 2003). Higher levels of H_2O_2 were also associated with the necrotic cell death in the type III necrosis exhibited in an interspecific wheat hybrid (Mizuno et al., 2010).

Hybrid Necrosis and Plant Immunity

The development of tissue lesions, wilting, tissue necrosis, the collapse of cells, and severely reduced fecundity are the main characteristics of hybrid necrosis in plants (Hermsen, 1963a, 1963b; Bomblies et al., 2007; Bomblies, 2009). In wheat, the first appearance of lesions due to hybrid necrosis appear at the tips of leaves (Sharma et al., 2003; Chu et al., 2006). Symptoms often resemble the hypersensitive resistance response of plants against pathogens (Chu et al., 2006; Mizuno et al., 2010), and tissue necrosis progresses from the tip to the base of the affected leaf before symptoms begin appearing in the next maturing leaf.

As proposed in the Dobzhansky-Muller (DM) model (Dobzhansky and Dobhansky, 1937; Muller, 1942; Bomblies and Weigel, 2007), divergent loci (DM loci) are involved in epistatic interactions that result in hybrid necrosis (Bomblies and Weigel, 2007). The exposure of plants to different pathogens and pests likely has promoted the selection for DM loci in plants. (Jones and Dangl, 2006; Chen et al., 2016). Since DM loci are comprised of plant defense-related genes, the plant genes and mechanisms

responsible for hybrid necrotic symptoms are possibly the same, or similar to those involved in defense responses to pathogens and pests. In Arabidopsis, an NB-LRR gene, which is a resistance gene also causes tissue necrosis (Bomblies et al., 2007). In wheat, the leaf rust resistant gene, *Lr13* was proposed to be the *Ne2m* gene, and a gene important to the development of hybrid necrosis (Zhang et al., 2016).

Gene expression studies in various plant species have shown the activation of genes which are also involved in disease response signaling during the development of hybrid necrosis (Bomblies et al., 2007; Bomblies, 2009). For example, pathogen response genes *PR1* and *PR5* were activated in interspecific tobacco hybrids produced from crosses between *Nicotiana suaveolens* Lehm. and *Nicotiania tabacum* (Masuda et al., 2007), and *Arabidopsis* hybrids which exhibited necrotic tissue showed an increased resistance to a broad-spectrum of pathogens compared with each parent (Bomblies et al., 2007). Another study using *A. thaliana* described the dependency of necrotic phenotypes on the expression of the plant defense hormone, salicylic acid (Alcazar et al., 2009). Mizuno et al. (2010) demonstrated the activation of defense genes during the expression of hybrid necrosis in interspecific hybrids between *T. turgidium* and *Ae. Tauschii*.; whereas, Zhang et al. (2014) reported that defense-related and stress-related genes were highly upregulated during the development of hybrid necrosis.

Effect of Temperature on Hybrid Necrosis

Temperature has a profound effect on the metabolic growth and development of plants (Mino et al., 2002; Stavang et al., 2007), and the type II symptoms of necrosis expressed in interspecific wheat hybrids was observed at low temperature (4^{o}C), but symptoms were suppressed when plants were grown at 23^{o}C (Mizuno et al., 2011). Conversely, wheat plants grown even at 30^{o}C did not exhibit reduced necrosis, and this response was attributed to a type III response induced by *Nec1* located to chromosome 7D (Mizuno et al., 2010). However, Dhaliwal et al. (1986) observed that intraspecific wheat hybrids grown at a high temperature did have reduced necrotic symptoms. Interspecific hybrids in tobacco grown at a high temperature (37^{o}C) did not exhibit an increase in ROS production, and in fact, the higher temperature suppressed lethality due to hybrid necrosis (Mino et al., 2002); whereas, in the same study, hybrids grown at 26^{o}C produced more ROS, and there was plant lethality due to necrosis. Interspecific lettuce (*Lactuca sativa* and *L. saligna*) hybrids possessing the necrosis gene *Rin4* had necrosis symptoms suppressed when hybrids were grown at 30^{o}C (Jeuken et al., 2009). As is the case with many disease response genes (Traw and Bergelson, 2010; Alcazar and Parker, 2011; Hua, 2013), expression of hybrid necrosis is dependent on temperature, a fact that only emphasizes that the same, or similar defense response genes are likely involved in the expression of plant hybrid necrosis.

Role of Purine Catabolism and Xanthine Dehydrogenase in Plants

Purine ring catabolism has an important role in plant nitrogen metabolism (Werner and Witte, 2011), and purine ring metabolism initiates with the conversion of adenosine monophosphate (AMP) to inosine monophosphate (IMP) by AMP deaminase (AMPD) (Fig. 1). From IMP, purine catabolism proceeds with the production of three compounds; hypoxanthine, xanthosine monophosphate (XMP), and guanosine monophosphate (GMP), which are the result of intermediate pathways in route to producing purine. All three pathways converge to produce the first common compound, xanthine (Zrenner et al., 2006), which in turn produces uric acid, allantoin, and allantoate. Oxidation of xanthine to form uric acid is catalyzed by xanthine dehydrogenase (XDH), which is before conversion of allantoin to allantoate. The enzyme XDH is also involved in the conversion of hypoxanthine to xanthine through the IMP pathway (Fig. 1). In the final step, CO_2 and NH_3 are released as the result of purine catabolism (Fig. 1), and the released NH_3 is re-assimilated through the glutamine oxoglutarate aminotransferase (GOGAT) pathways (Zrenner et al., 2006; Werner and Witte, 2011).

Thus, the purine catabolism pathway depends on XDH (Fig. 1). *Arabidopsis thaliana* mutants lacking XDH showed reduced growth, reduced fertility, and premature senescence (Nakagawa et al., 2007; Brychkovaet al., 2008). Application of exogenous uric acid to XDH mutant plants enabled the plants to develop more normally (Nakagawa

et al., 2007), which illustrated the important role of XDH in purine ring catabolism and nitrogen re-assimilation in plants. Similarly, Atkins et al. (1988) working with soybean (Glycine max) demonstrated the important role of XDH in the synthesis of uric acid, and subsequent nitrogenase activity for normal root nodule growth and development. In addition to its role in purine catabolism, XDH has been linked to superoxide production during stress responses to drought, or pathogens (Montalbini, 1995; Hesberg et al., 2004).

Allopurinol as an XDH Inhibitor

Allopurinol [4-hydroxypyrzolo (3, 4-d) pyrimidine] is a synthetic analogue of hypoxanthine (Elion et al., 1963). It is an inhibitor of xanthine oxidase and XDH (McCollister et al., 1964; Massey et al., 1970). Its inhibitory effect on xanthine oxidase has been employed in the treatment of the human conditions of gout and hyperuricaemia (Krenitsky et al., 1967; Montalbini and Della Torre, 1996). In plants, along with inhibiting XDH activity, allopurinol undergoes a conversion (Krenitsky et al., 1967). It is converted to oxipurinol by XDH and the oxipurinol inhibits XDH activity (Della Torre and Montalbini, 1995)(Fig. 2A). Purine nucleoside phosphorylase converts allopurinol and oxipurinol and to their respective allopurinol and oxipurinol ribonucleosides (Krenitsky et al., 1967; Della Torre and Montalbini, 1995: Fig. 2B). As more ribonucleosides are produced, less allopurinol is available for the inhibition of XDH (Della Torre and Montalbini, 1995). The application of allopurinol to bean (*Phaseolus*

vulgaris), broad bean (*Vicia faba*), and tobacco resulted in the production of ribonucleosides; whereas, in wheat, there is negligible production of nucleosides, which enables exogenously applied allopurinol to primarily inhibit XDH. The allopurinol concentration required for the inhibition of XDH in wheat is five times lower than that required to achieve the same level of XDH inhibition in bean and broad bean (Montalbini 1992a, 1992b; Della Torre and Montalbini, 1995).

As an effective inhibitor of XDH, allopurinol has been employed to study the role of XDH in plants. In soybean, the use of allopurinol inhibited uric acid synthesis, and ultimately resulted in nodule senescence (Atkins et al., 1988). Inhibition of XDH has also resulted in plants that are susceptible to pathogens when they normally exhibit a resistant phenotype in the presence of pathogens, or pests (Montalbini, 1992a, 1992b; Adam et al., 2000).

Plant Responses during Stress Conditions

Plants exposed to biotic and abiotic stresses exhibit disrupted metabolic activity, with the most profound effects being a disruption of some basic physiological processes (Heil and Bostock, 2002; Rejeb et al., 2014). During pathogenesis, receptor-like kinase (RCLs) and receptor-like proteins (RCPs) interact with pathogens to induce microbe-associated molecular patterns (MAMP) or pattern-associated molecular patterns (PAMPs) (Padmanabhan et al. 2009; Greeff et al. 2012). Similarly, R proteins from NB-LRR

(Nucleotide binding – leucine rich repeat) groups of genes serve as receptors for the pathogen effectors (Nishimura and Dangl, 2010; Meng and Zhang, 2013). Recognition of PAMPS initiates the PAMPs-triggered immunity (PTI), and in the same way, recognition of pathogen effectors results in effector-triggered immunity, eventually leading to hypersensitivity responses, and cell death (Coll et al., 2011; Meng and Zhang, 2013).

After plant recognition of a pathogen or pest, defense responses are regulated by a complex network of mitogen activated protein kinases (MAP kinases), ROS bursts, signaling hormones, and transcription factors, all of which result in a reprograming of gene expression (Meng and Zhang, 2013). MAP kinases are active downstream of the receptors/sensors of stresses, and they act on the extracellular stimuli to develop an intracellular response (Meng and Zhang, 2013). Some MAP kinases specifically regulate the signaling of downstream abiotic stress defense responses, while some are specific for pathogen resistance downstream responses, while yet others are responsible for responses to both biotic and abiotic stresses (Fraire–Valazquez et al., 2011; Meng and Zhang, 2013). There is some crosstalk between MAP kinases and ROS signaling during plant response to stress (Saul et al., 2011; Meng and Zhang, 2013; Rajeb et al., 2014). Defense-related MAP kinases (e.g., MAP kinase3, MAP kinase6, MAP kinase4, MAP kinase9, MAP kinase12) can be activated with the exogenous application of H_2O_2, suggesting that MAP kinases work downstream of ROS generation during the cascade of defense reactions (Pitzschke and Hirt, 2009; James et al., 2009). However, there are other reports

documenting an upstream action of MAP kinases during a response to stress (Ren et al., 2002; Meng and Zhang et al., 2013), and MAPK-activated ROS generation is independent of NADPH oxidase-generated ROS (Liu et al., 2007).

Like MAP kinases, an increase in Ca^{2+} production also has an important role in the plant response to an abiotic, or biotic stress. Activation of calcium-dependent kinases (CDP Kinases), calreticulins and calmodulins causes the transient increase in cytosolic Ca^{2+} (Mahajan and Tuteja, 2007; Cheval et al., 2013). Both MAP kinases and CDP kinases could be activated in the same stress response, and CDP Kinases and MAP kinases may regulate different pathways in response to stresses (Wurzinger, 2011).

During plant stress responses, transcriptional factors act downstream of MAP kinases and Ca^{2+} signaling (Meng and Zhang, 2013; Jalmi and Sinha, 2015). They modulate the transcription rate of target genes in response to environmental conditions (Pandey and Somssich, 2009; Ambawat et al., 2013). The majority of WRKY activate during pathogen infestation, as do many of the myeloblasts (MYB), basic leucine zipper (bZIP), C–repeat binding factors (CBF), and NAC transcription factors (NAM; no apical meristem: ATAF; Arabidopsis transcription activation factor: and CUC; cup-shaped cotyledon) (Ambawat et al., 2013; Rajeb et al., 2014). Many of these transcription factors activate and perform multiple functions during both abiotic and biotic stresses (Fraire-Velázquez et al., 2011; Rajeb et al., 2014).

Downstream of the cascade of defense reactions, plant hormones can regulate the responses to a wide range of biotic and abiotic stresses (Bari et al., 2009). For example, abscisic acid (ABA) is a main driver for the regulation of abiotic stress responses (Xiong et al., 2002; Cramer et al., 2011); whereas, salicylic acid, jasmonic acid (JA), and ethylene are often associated with modulating biotic stresses as part of the overall stress regulation network (Liu et al., 2008; Bari et al., 2009). The eventual outcome of this cascade of defense reactions is the reprogramming of genes that result in the production of secondary compounds in response to specific environmental conditions. Plants produce lignins, phenylpropanoids, phenolics, flavonoids, and many other secondary compounds with antioxidant properties during exposure to biotic and abiotic stress environments (Akula and Ravishankar, 2011; Ebrahim et al., 2011; Tian et al., 2015).

Transcriptome Analysis

The transcriptome reflects all expressed genes at a specific developmental, or physiological stage of plant development. An understanding of gene expression is crucial to knowing how they are upregulated in response to the need to engage specific physiological processes (Wang et al., 2009).

Microarray technology has developed rapidly as a means to analyze gene expression in plants. The technology makes use of fluorescently labeled cDNA that is subsequently hybridized to a microarray chip, with the results afterwards interpreted from

this hybridization. Microarray relies on *a priori* genome sequence knowledge, and microarray probes are prone to cross-hybridize with probes of background platforms (Okoniewski and Miller, 2006; Royce et al., 2007), detection and accurate quantification of low gene expression can be difficult. Furthermore, the results are of this gene expression are not reproducible across different environments (Shendure, 2008). Conversely, sequence-based approaches directly determine the cDNA sequence. Sanger sequencing of cDNA, or expressed sequence tag [EST] libraries can be used to more directly assess gene expression (Wang et al., 2009). The tag-based methods of Sanger sequencing, i.e., serial analysis of gene expression (Velculescu et al., 1995; Gerhard et al., 2004), cap analysis of gene expression (Shiraki et al., 2003; Kodzius et al., 2006,), and massively parallel signature sequencing (Brenner et al., 2000; Peiffer et al., 2008; Kaushik, et al., 2008) are high throughput methods that provide great precision in determining gene expression; however, these methods are expensive, and short tags cannot be quantified properly in the reference genome (Wang et al., 2009).

RNA-Seq

RNA–seq has provided an unprecedented opportunity to comprehensively analyze entire plant transcriptomes (Wang et al., 2009; Conesta et al., 2016), and RNA–sequencing enables the identification of novel transcripts that are specific to the individuals, or populations of interest. High resolution identification and accurate

quantification of transcripts present in the transcriptome are key to producing useful information (Wolf, 2013), and the specificity and sensitivity of RNA sequencing are better than that of previously used transcriptome analytical methods (Wang et al., 2009; Mortazavi et al., 2008).

The development of quick and cost effective next generation sequencing (NGS) technology has revolutionized the study of the plant genome (Mardis, 2008) because sequence data can be rapidly acquired and compared with transitional sequencing (Strickler et al., 2012). Also, complementary computational bioinformatics tools have been developed to accurately quantify the sequencing data, which contributes to offering a high-throughput method for genomics studies (Conesta et al., 2016). The study of plant gene function, or transcriptomics has greatly benefitted from the development of these sequencing technologies (Wang et al. 2009; Conesa et al., 2016).

The NGS platforms that are now available include; the Roche/454 FLX Pyrosequencer, the Illumina Genome analyzer, the Applied Biosystems SOLiD™ sequencer,the Helicos Helicope™, and Pacific Biosciences SMRT (Mardis, 2008, Fullwood 2016).

The Illumina-based method uses polymerase-based sequence-by-synthesis to provide accurate sequence information (Mardis, 2008), and this method has been gaining in use. The Illumina work flow starts with the random annealing of the DNA library to the surface of the flow cell. Adaptors are attached to the DNA molecules that enable

bridge amplification and cluster formation on the flow cell after amplification. This approach permits each cluster to be supplied with the polymerase, and four differentially labeled nucleotide bases. Imaging of the flow cell after each cycle of sequencing allows for the specific addition of bases to be monitored. Repetition of this process culminates with the determination of a complete sequence (Mardis, 2008).

The standard RNA-seq procedures are often changed and adapted to the species being analyzed and to the objectives of an experiment. The general procedures are; production of millions of short sequence reads, followed by mapping, assembling, normalizing, and testing the sequence for differential gene expression (Wang et al., 2009; Conesta et al., 2016). The RNA samples are typically fragmented and converted to cDNA to produce a nucleotide library that is sequenced by NGS. The reads generated are assembled and aligned onto a reference genome. If a reference genome is not available, transcripts are constructed from the reads, and there is *de novo* assembly (Wang et al., 2009; Wolf, 2013). Bioinformatics software has been developed, which can be used to map the reads, and accurately quantify transcripts identified during the RNA–seq analysis (Jean et al., 2010; Wang et al., 2010; Wu and Nacu, 2010; Grabherr et al., 2011; Haas et al., 2013; Xie et al., 2014).

Differential Expression Analysis

An important feature of RNA-seq is the analysis of gene expression (Wang et al., 2009). RNA-seq quantifies the number of reads mapped to a gene or transcripts, and the number of reads are normalized based on the reads per kilobase exon model per million mapped reads (RPKM), fragments per kilobase of exon model per million mapped reads (FPKM), or based on the transcripts per million (TPM). Quantification of differentially expression transcripts is performed (Strickler et al., 2012; Conesa et al., 2016), and the differential expression of genes, or genes product over treatments provides some insight as to the genes and gene mechanisms that may be important in certain plant responses (Wang et al., 2009; Strickler et al., 2012).

Significance of the Present Study

Hybrid necrosis poses a barrier to producing desirable wheat parental combinations, but yet the same, or similar plant stress response genes are often implicated as being the cause of this necrosis (Bomblies and Weigel, 2007; Mizuno et al., 2010). Wheat hybrid necrosis has received relatively little attention, particularly as relates to what genes and gene mechanisms are functioning at the molecular level, and as relates to how these mechanisms compare to those involved in responding to stresses (Bomblies and Weigel, 2007). Since as a polyploidy, bread wheat possesses multiple genomes and is

expected to express hybrid necrosis more frequently than other crops, this underscores the need to more thoroughly understand the mechanisms and processes involved. Additionally, studying wheat hybrid necrosis could lead to a better understanding of the responses of plants to pathogens and pests given that plant cell death and subsequent tissue necrosis appears to be common to both processes

Several reports suggest that ROS triggor programmed cell death (PCD) in hybrid necrosis (Khanna–Chopra et al., 1998; Sharma et al., 2003). In developing future breeding strategies as well as integrated plant management strategies, it is important to fully understand the potential role of ROS in necrosis development, as well as how XDH functions to impact hybrid necrosis in wheat.

Though the epistatic interaction of *Ne1* and *Ne2* is necessary to express hybrid necrosis in wheat, the degree of necrosis apparently differs according to the specific hybrid allelic combination achieved (Hermsen, 1963a, 1963b; Zeven, 1972; Takumi et al., 2013). Few of the *Ne1* allele variants (eg., $Ne1^{w}$ - weak, $Ne1^{m}$ – moderate, and $Ne1^{s}$ - strong alleles) and few of the *Ne2* allele variants (eg., $Ne2^{w}$ - weak, $Ne2^{mw}$ - moderate to weak, $Ne2^{m}$ - moderate, $Ne2^{ms}$ - moderate to strong, and $Ne2^{s}$ - strong alleles,) associated with different levels of tissue necrosis have been validated (Hermsen, 1963a; Chu et al., 2006; Takumi et al., 2013). A more detailed characterization of the *Ne* genes, and their allelic variants is expected to help in the identification and development of closely linked markers to assist breeders avoid producing unwanted hybrid combinations.

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Fig. Legends

Fig. 1. Plant purine catabolism and enzyme XDH (xanthine dehydrogenase) pathway to conversion of hypoxanthine to xanthine and xanthine to uric acid. AMP = adenosine monophosphate, IMP = Inosine monophosphate, GMP = guanosine monophosphate. The final products of purine catabolism are NH_3, CO_2, and glyoxylate. The end products are reused in photosynthesis, and in nitrogen reassimilation (Adapted from Zrenner et al., 2006; Werner and Witte, 2011).

Fig. 2. Xanthine dehydrogenase (XDH) inhibition by allopurinol and XDH mediated allopurinol oxidation. B). Purine nucleoside phosphorylase mediated allopurinol and oxipurinol ribosidation (Adapted from Krenitsky et al., 1967; Della Torre, and Montalbini, 1995)

Figures

Fig. 1.

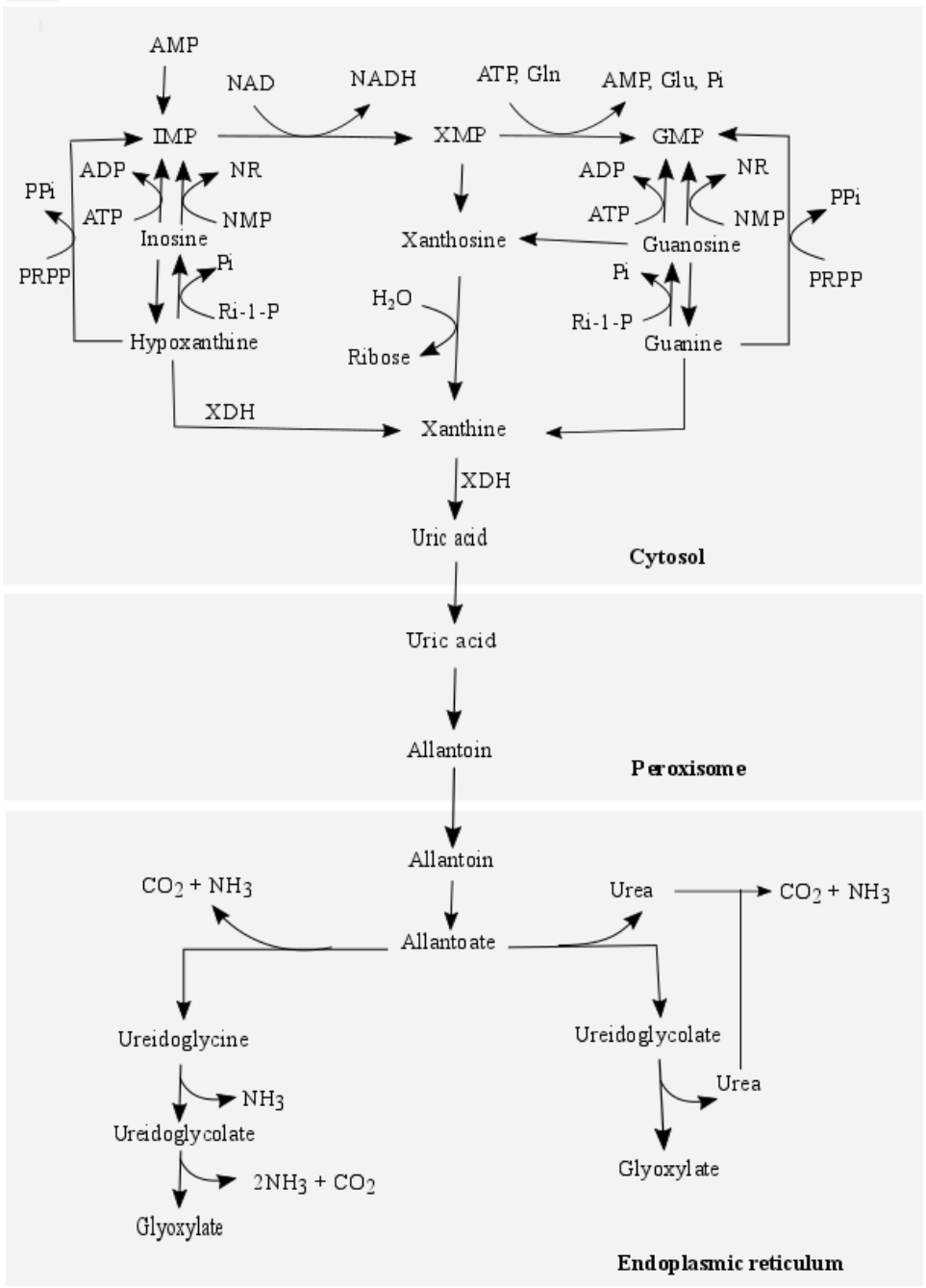
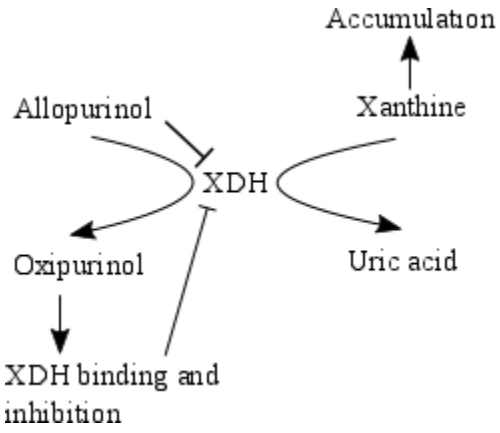
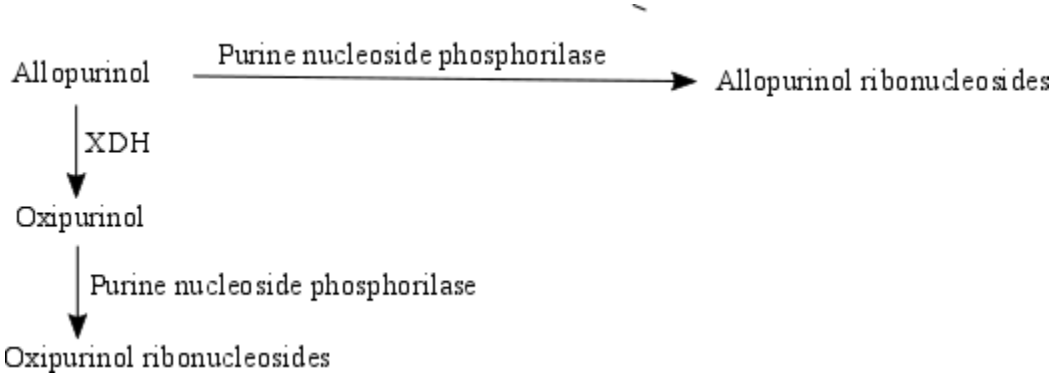


Fig. 2.

A


Accumulation
Allopurinol
Xanthine
XDH
Oxipurinol
Uric acid
XDH binding and
inhibition

B.


Allopurinol
Purine nucleoside phosphorilase
Allopurinol ribonucleosides
XDH
Oxipurinol
Purine nucleoside phosphorilase
Oxipurinol ribonucleosides

CHAPTER 2: The Role of Reactive Oxygen Species, Xanthine Dehydrogenase, and Temperature in the Expression of Hybrid Necrosis in Wheat (*Triticum aestivum* L.)

Abstract

Hybrid necrosis in wheat (*Triticum aestivum* L.) is characterized by the premature death of leaves or plant tissue in certain parental combinations. It is caused by the interaction of two dominant complementary genes, *Ne1* and *Ne2*, located to chromosomes 5B and 2B, respectively. Reactive oxygen species (ROS) may be important to the programmed cell death component of hybrid necrosis. Objectives of this experiment were; to 1) determine the levels of H_2O_2, 2) determine the activity levels of xanthine dehydrogenase (XDH), and 3) determine the effect of temperature changes in F_1 hybrids exhibiting hybrid necrosis. Germplasm used consisted of 'Alsen', and synthetic hexaploid wheat lines known to exhibit hybrid necrosis. The materials were planted and tested in both greenhouse and growth chamber environments. The level of H_2O_2 production during development of necrosis in F_1 hybrids is not significantly higher, than the level observed in the parental lines not exhibiting necrosis. This suggests that H_2O_2-mediated programmed cell death is not responsible for the symptoms of necrosis in these wheat hybrids. However, other ROS and their levels during the expression of necrosis were not measured, so the role of ROS as an important component of hybrid necrosis can not be completely dismissed. Soil application of allopurinol to inhibit XDH activity

resulted in only a slight reduction in ROS generation, as measured in some of the tissue samples, and it resulted in reduced growth and grain yields for hybrids in one greenhouse experiment. These results suggest that XDH-induced ROS does not substantially contribute to the development of necrosis; whereas, the impact on plant performance in one experiment likely emphasizes the importance of XDH to purine ring catabolism, and plant nitrogen assimilation. Complete suppression of necrosis development in F_1 plants grown at 30^{O}C, and enhanced necrosis at 23^{O}C likely means that *Ne1* and *Ne2*, and the response that results from their interaction is temperature-sensitive.

Introduction

Hybrid necrosis in wheat (*Triticum aestivum* L.) is represented by the premature death of seedlings, leaves, or plants in specific parental combinations (Hermsen 1963a). The complementary interaction of two dominant genes (*Ne1* and *Ne2* located on chromosomes 5BL and 2BS, respectively) causes lethality, or severely reduces plant productivity in wheat hybrids (Tsunewaki, 1960; Hermsen 1963a, 1963b; Nishikawa, 1974; Chu et al., 2006). Wheat genotypes having either *Ne1* or *Ne2* genes are widely distributed throughout the world, and this can limit the use of certain combinations of parents from diverse germplasm pools in order to combine traits in the development of improved genotypes (Pukhalskiy et al., 2000, 2009; Vikas et al., 2013).

The importance of ROS are often emphasized in the expression of hybrid necrosis. Superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) are the major forms of ROS (Mehdy, 1994; Apel and Hirt, 2004; Hung et al., 2005), and ROS may serve to directly repel, or block plant pathogens and pests, or serve as signal molecules to cause programmed cell death of plant tissue (Apel and Hirt, 2004, Liu et al., 2010). The hypersensitive response is a result of programmed cell death that is accompanied by the rapid accumulation of ROS at the pathogen-host interaction site, and hypersensitivity and cell death represent typical plant responses to pathogens and pests (Apostol et al., 1989; Heath, 2000; Mur et al., 2008). Hypersensitive response-like lesions are also produced by necrotic wheat hybrids (Chu et al., 2006). Higher levels of O_2^-, and H_2O_2 have been observed before and during the progression of leaf tissue necrosis in such hybrids (Khanna-Chopra et al., 1998; Sharma et al., 2003; Mizuno et al., 2010). Transcriptome analysis of wheat and several other plant species has identified the up-regulation of defense/immune-related genes (Bomblies and Weigel, 2007; Jeuken et al., 2009; Alcázar et al., 2010; Mizuno et al., 2010; Zhang et al., 2014). Thus, these reports suggest that hybrid necrosis is a result of programmed cell death (PCD), and in the absence of any pathogen or pest, it has been proposed that it is something of an autoimmune response by the plants expressing the necrosis (Bomblies et al., 2007).

Antioxidant enzyme activities are involved with the detoxification of ROS, and insufficient production of antioxidants will enable toxic ROS to build up in plants, which

often leads to the production of oxidative stress symptoms (Apel and Hirt, 2004; Tripathy and Oelmuller, 2012). Higher activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase have been documented during hybrid necrosis in wheat (Dalal and Khanna-Chopra, 2001). Activation of these antioxidant enzymes suggests that there is a higher production of ROS during hybrid necrosis. On the other hand, in the same study (Dalal and Khanna-Chopra, 2001) there was a low level of catalase activity. Superoxide dismutase converts superoxide to H_2O_2, and ascorbate peroxidase, glutathione reductase, and catalase are involved in detoxifying H_2O_2 (Montalbini, 1992a, 1992b; Apel and Hirt, 2004).

Purine metabolism in plants is a key part of plant nitrogen metabolism (Zrenner et al., 2006; Werner and Witte, 2011). While Xanthine is the first common intermediate compound in the purine ring catabolism (Zrenner et al., 2006), Xanthine dehydrogenase (XDH) is critical for the oxidation of xanthine to uric acid, leading to the oxidation of hypoxanthine to xanthine through the inosine monophosphate (IMP) pathway (Zrenner et al., 2006; Watanabe et al., 2014). After uric acid formation, uricase converts the uric acid to the nitrogenous compound allantoin. Ultimately CO_2 and NH_3 are released from allantoin as the final products of purine catabolism (Todd and Polacco, 2004; Zrenner et al., 2006). These end products are recycled as CO_2 for photosynthesis, and NH_3 is re-assimilated through the glutamine oxoglutarate aminotransferase (GOGAT) pathway (Zrenner et al., 2006). Thus, XDH activity not only catalyzes purine catabolism but also

assists in the reassimilation of nitrogen in the plant. *Arabidopsis* XDH mutants exhibit reduced growth, reduced fertility, and premature senescence (Nakagawa et al., 2007; Brychkova et al., 2008; Werner and Witte, 2011). Similarly in soybean, inhibition of XDH prevented the formation of allantoin, which resulted in reduced plant growth and N_2 fixation in soybean (Atkins et al., 1988).

In addition to its role in purine catabolism, XDH is involved in responses to stress, drought, and several pathogen-host interactions (Montalbini, 1992a, 1992b; Hesberg et al., 2004; Yesbergenova et al. 2005; Watanabe et al., 2010). Xanthine dehydrogenase activity is tied to the production of superoxide, which is converted to the more-stable H_2O_2 by superoxide dismutase (Montalbini, 1992a, 1992b; Hesberg et al., 2004). Allopurinol, has been successfully employed to inhibit the activity of XDH (Montalbini, 1992a,1992b; Berner and Van der Westhuizen, 2010). Additionally, allopurinol can inhibit XDH-induced ROS production (Berner and Van der Westhuizen, 2010). There is evidence that plant responses to rust pathogens can be changed as a result of allopurinol treatments, and presumably because of the concomitant inhibition of ROS (Montalbini, 1992b; Adam et al., 2000).

Temperature can significantly impact plant metabolic activity (Stavang et al., 2007), and as an example, hybrid necrosis symptoms in lettuce, caused by *Rin4*, were not observed when plants were grown at a constant 30^{O}C (Jeuken et al., 2009). The type II hybrid necrosis symptoms caused by *Net2* develop only under low temperature in

interspecific hybrids between tetraploid wheat and *Aegilops tauschii*, (Nishikawa, 1962b; Mizuno et al., 2011). However, gene function in the expression of necrotic symptoms can be independent of temperature. For example, expression of type III necrosis symptoms as determined by *Nec1* in interspecific hybrids between tetraploid wheat and *Ae. tauschii* was unaffected by a similar changes in temperature (Mizuno et al., 2010). Also, intraspecific hybrids of *T. aestivum* (WL711/C306 and WL711/PBW34) that typically exhibit hybrid necrosis symptoms did not develop symptoms while growing at 28^{o}C (Dhaliwal et al., 1986). Similarly, high temperature can led to detoxification of ROS, or inhibited generation of ROS, which suppresses tissue necrosis and prevents of the cell death of interspecific tobacco hybrids (Mino et al., 2002).

Though an autoimmune type of response has been described as the cause of hybrid necrosis in plants, the physiological changes associated with the response are poorly understood (Bomblies et al., 2007; Bomblies and Weigel, 2007; Mizuno et al., 2010; Zhang et al., 2016). Additionally, though cell death in hybrid necrosis is likely a consequence of ROS production (Khanna-Chopra et al.,1998; Sharma et al., 2003; Mizuno et al., 2010), the concept needs to betested more broadly in its application to wheat. Given the importance of XDH to purine catabolism and its role in biotic and abiotic stress responses (Montalbini, 1992a, 1992b; Hesberg et al., 2004; Watanabe et al., 2014), it is logical to predict it could have a similar level of importance to the response symptoms expressed in hybrid necrosis. Hybrid necrosis has been expressed by F_1

hybrids produced from crosses between the hexaploid wheat cultivar ‘Alsen’ and two synthetic hexaploid wheats ‘TA4152-37’ and ‘TA4152-19’ (Chu et al., 2006), and therefore the objectives of this study were to 1) determine if ROS levels were also elevated during the expression of hybrid necrosis,2) determine if XDH activity increased during expression of hybrid necrosis, and 3) determine if changes in temperature affect the expression of hybrid necrosis in wheat.

Materials and Methods

Plant Material

The parental lines used to produce hybrids expressing necrosis and those included in the experiments included the hard red spring wheat variety ‘Alsen’ [pedigree: ND674//ND270/ND688, where ND270 = ND2603(Sumai3/Wheaton)/Grandin] (Frohberg et al., 2004, 2006) released by the North Dakota Agricultural Experimental Station, and the synthetic hexaploid wheat lines ‘TA4152-37’ [pedigree: 68.111/RGB-U//Ward/3/FGO/4/RABI/5/Ae. tauschii(878)] and ‘TA4152-19’ [pedigree: Dverd 2/Ae. tauschii (221)] (Chu et al., 2006) developed by CIMMYT. Alsen was the male parent in the production of hybrids with ‘TA4152-37’ and ‘TA4152-19’ (Chu et al., 2006). Thus, there were three parental genotypes (‘Alsen’, ‘TA4152-37’, and ‘TA4152-19’) and two F_1 hybrids (‘TA4152-37/Alsen’ and ‘TA4152-19/Alsen’) that comprised the genotypes included in experiments.

Three greenhouse experiments were conducted at South Dakota State University (SDSU) located at Brookings, SD, USA. The first such experiment was conducted in the off-campus Seed Technology Building greenhouses. Greenhouse environmental conditions consisted of a constant 20^{o}C ± 3 temperature, with a 16 h light period maintained by supplemental lighting. The second and third greenhouse experiments were conducted in the on-campus spring wheat breeding greenhouse located at SDSU. The conditions in this greenhouse consisted of a constant temperature of 25^{o}C ± 3, with a 16 h light period maintained by supplemental lighting.

Whereas all five genotypes were tested in the first greenhouse experiment, only three ('Alsen', 'TA4152-37', and their F_1 progeny) were included in second and third greenhouse studies. Genotypes in all greenhouse experiments were planted and arranged to a RCB design with the first experiment including 5 replications, the second including a minimum of 9 replications, and the third experiment including 6 replications.

All three greenhouse experiments were conducted with four plants grown in 17 cm x 15 cm plastic pots filled with Sunshine Mix 1 (Sun Gro Horticulture Canada Ltd., Seba Beach, AB, Canada). After emergence, plants either received, or did not receive an aqueous solution of allopurinol as a soil drench treatment. Plants that did not receive allopurinol represent non-treated controls. In the first experiment, four different allopurinol concentrations (0 μM, 15 μM, 30 μM, and 50 μM) were prepared and applied as a soil drench. Based on the results derived from this experiment, three (0 μM, 15 μM,

and 30 μM) and two (0 μM and 50 μM) allopurinol treatments were applied in the second and third experiments, respectively. Initially, 300 ml of each treatment was applied per pot and administered on two alternate days. At a later stage of plant growth, a greater volume of solution was applied since additional irrigation was required. In total, soil allopurinol treatments were made 16 times in both the first and second experiments, while plants in the third experiment received a total of 7 allopurinol treatments.

Temperature Sensitivity Experiment

Three wheat genotypes; 'Alsen', 'TA4152-37' and the F_1 plants from 'Alsen' and 'TA4152-37' (pedigree = TA4152-37/Alsen) were grown at 15^{o}C, 23^{o}C and at 30^{o}C in two growth chambers, with a 14 h supplemental light period. Temperature experiments were conducted in Conviron (CMP 3244, Conviron, and Winnipeg, Canada) and Percival scientific (Percival Scientific, Inc., Perry, IA) growth chambers, with plants grown in 10 cm x 10 cm plastic pots. The plant growth period was 49 d, and experiments were arranged to a RCB design with a minimum of 10 replications.

Necrosis Measurements

Necrosis measurements were recorded for plants grown in the second greenhouse experiment, and the experiments conducted in the growth chambers. Eleven days after leaf emergence, the first necrotic lesions were observed at the tip of the fifth leaves on the

hybrid plants in the greenhouse experiment. Progression of necrosis was quantified using image analysis. Whole leaf images were taken with a DSLR camera (New York, USA, Canon, Inc.) at two time points. In the second greenhouse experiment, the first image was taken at 14 d post-emergence and the second at 17 d post-emergence. In the temperature sensitivity experiments, the first and second images of leaves were taken at 14 and 19 d post-emergence, respectively. Assess 2.0 software (Lamari, 2008) was used to analyze the digital leaf images and calculate the percent leaf necrosis.

H_2O_2 Measurements

In the second and third greenhouse experiments, H_2O_2 levels were measured in the leaf tissues in both allopurinol treated (30 μM) and non-treated (0 μM) plants. Since the accumulation of ROS has been associated with the expression of necrosis, measurements of H_2O_2 levels were made based on the necrosis progression observed in leaves. Two measurements were made using the first and fifth leaves; whereas, only a single measurement was taken from the second leaves.

An Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes, Eugene, OR, USA) was used to measure H_2O_2 accumulation in plant leaves. Harvested whole leaves were frozen immediately in liquid N, and were later homogenized to a fine powder. Each 50 mg sample was mixed with 400 µL phosphate buffer (20 mM K_2HPO_4, pH 6.5). The resulting homogenate was centrifuged at 12,000 rpm for 20 minutes at 4^{o}C. After

centrifugation, 10 µL supernatant was collected and mixed with 100 µM of the Amplex Red reagent (10-acethyl-3, 7-dihydrophenoxazine) and 0.2 U/ml horseradish peroxidase in a 96 well flat bottom plate (Sigma–Aldrich, St. Louis, MO). The mixture was kept in darkness at room temperature for 30 min. A BioTek Microplate Reader (Synergy HT Bio-Tek, Winooski, VT) was used to measure the fluorescence (excitation at 540 nm, emission at 620 nm) of each sample. Gene5 software was used to quantify ROS levels using a standard curve constructed using known concentrations of H_2O_2.

Leaf Chlorophyll Measurements

A Spad-502 meter (Konica-Minolta, Japan) was used to measure the chlorophyll content in leaves. Leaves were collected from the second (third and eighth leaves of the plants) and third greenhouse (third leaves only) experiments, and in the temperature sensitivity experiement (third leaves only) were measured. Chlorophyll measurements were taken 13 d post leaf emergence when necrosis was first observed in the F_1 plants. The second measurement was taken 18 d post leaf emergence. Six Spad-502 readings were taken from each leaf.

Plant Height and Yield Measurements

In the first and second greenhouse experiments, plant height and grain yield measurements were recorded. Harvested grain was dried at 37^{O}C for 24 h to achieve a standard moisture content before weighing.

Statistical Analysis

An analysis of variance (ANOVA) was calculated to determine any significant difference among and between genotypes and treatments. Percent necrosis data from greenhouse and growth chamber experiments were transformed using a square-root transformation in order to meet the assumptions of normality. The Proc GLM procedure in SAS (SAS Institute, 2011) was run to calculate the ANOVA. Means were separated using least significant difference (LSD), and significance differences were determined at alpha equal to 0.05.

Results

Development of Necrosis

Mean necrosis measurements between the first and second measurement times were significantly different (Table. 1), but allopurinol treatments did not have a significant effect on the development of necrotic symptoms. At 14 d after leaf emergence, the mean range in leaf necrosis was 12% to13.5% of the leaf area, whether plants were treated with allopurinol, or not. The second necrosis measurement, taken 3 d later, was

characterized by a significant increase in necrotic tissue development in all allopurinol treated and non-treated F_1 plants (Fig. 3). Over 3 d, the necrotic lesion area progressed from means of 12% to13.5% to means of 43.3 to 49.8% (Fig. 3).

H_2O_2 Accumulation in Leaves

Second Greenhouse Experiment

Genotype effects were significant (Table 2; Fig. 4A), as was the date for measurements (Table 2) and the genotype x date interaction (P<0.0001, Table 2) for H_2O_2 levels measured from the fifth leaf. Allopurinol treatments did not have a significant effect on the levels of H_2O_2. At the onset of necrosis in the fifth leaf, all three genotypes exhibited significant differences in the levels of H_2O_2 in leaves. During the onset of necrotic symptoms in the leaves of hybrids, H_2O_2 levels were intermediate to those observed in the parents, whether they were treated with allopurinol, or not (Fig. 4A). At the point when a second measurement was made, leaf necrosis was greater than 35% of the total leaf area in the hybrids, but levels of H_2O_2 in the leaves of the hybrids were less than those in the parents (Fig. 4A).

Third Greenhouse Experiment

The H_2O_2 measurements from the first leaves of plants were significantly different if genotypes were treated with allopurinol (Table 2). The genotype and genotype

x date interactions were both significant for levels of H_2O_2 (Table 2). Allopurinol treatments did not inhibit the production and accumulation of H_2O_2 in the hybrid plants, and levels were comparable to those expressed by the‘Alsen’ parent. However, H_2O_2 levels in the hybrids were significantly higher than those for the female parent ‘TA4152-37’. When the necrotic leaf area was approximately 25% in the hybrids, there was a significant decrease in H_2O_2 in the hybrid and in both parental genotypes (Fig. 4B). The H_2O_2 level in F_1 hybrid plants at the time of the second measurement (~25% leaf necrosis) was similar to that of the female parent, but significantly lower than that observed in ‘Alsen’. Treatment with allopurinol reduced the H_2O_2 content in the first leaves sampled,which was significant in both ‘TA4152-37’ and the hybrid, as it was in ‘Alsen’ when necrotic tissue reached approximately 25% of the total leaf area(Fig 4B).

Wheat genotype was significant for H_2O_2 levels measured form the second leaves but there was no significant effect of the allopurinol treatment (Table 2). Regardless of allopurinol treatment, the H_2O_2 levels in leaf tissue collected from the hybrids were intermediate to those from leaves of the two parents (Fig. 4C).

Leaf Chlorophyll Content

Second Greenhouse Experiment

In the second greenhouse experiment, allopurinol treatments had a significant effect on leaf chlorophyll content as sampled from the eighth leaf (P <0.0001, Table 3);

however, treatments did not have an effect on chlorophyll content as sampled from the third leaf. Chlorophyll readings taken at 13 and 18 d after leaf emergence were significantly different for genotype [third leaf (Table 3; Fig. 5A) and eighth leaf (Table 3; Fig. 5B)]. Significant differences were also calculated between measurements taken at two different dates, as sampled from the eighth leaf (Table 3)], and the interaction of genotype x date was significant (Table 3).

All three genotypes exhibited significant differences in chlorophyll content. Leaves of the parental line 'TA4152-37' had the highest concentration of chlorophyll, while hybrid plant leaves had the least chlorophyll.

Interaction effects of allopurinol treatments with genotypes and sampling times of necrosis were not significant. Allopurinol treatments had no significant impact on chlorophyll content (Fig. 5A). 'Alsen' and F_1 plants treated with allopurinol exhibited higher chlorophyll concentrations than non-treated plants of the same genotype (Fig. 5B). There was no significant difference in leaf chlorophyll content between the genotypes treated with 15 µM and 30 µM allopurinol. Irrespective of allopurinol treatment, chlorophyll content was significantly lower in the leaves of the hybrid plants as necrosis progressed. Chlorophyll measurements taken from F_1 plant leaves 18 d after leaf emergence were significantly lower than those taken 13 d after leaf emergence (Fig. 5A and 5B).

Third Greenhouse Experiment

The effect of genotype on chlorophyll content was apparent in the measurments taken on the third leaves (Table 3). The date of the measurement was significant (Table 3), as was the genotype x date interaction (Table 3) (Fig. 5C). Allopurinol treatment effects were not significant, and as in the second experiment, the hybrid plants lost chlorophyll from their leaves as necrosis progressed.

Plant Height

First Greenhouse Experiment

The genotype effect was significant for plant height (Table 4; Fig. 6A). Allopurinol treatments reduced plant height (Table 4; Fig. 6A), but the allopurinol x genotype interaction was not significant. Allopurinol-treated hybrid plants ('TA4152-37/Alsen' and 'TA4152-19/Alsen') and the female parent plants ('TA4152-37') were significantly shorter than their corresponding control plants (Fig. 6A).

Second Greenhouse Experiment

Three wheat genotypes 'TA4152-37, 'Alsen' and 'TA4152-37/Alsen' differed in plant height and the genotype effect for plant height was significant (Table 4; Fig. 6B). There was no significant treatment effect for allopurinol, or genotype x treatment interaction effect for plant height (Fig. 6B).

Grain Yield

First Greenhouse Experiment

The genotype effect was significant for grain yield (Table 5), as was the allopurinol treatment effect (Table 5; Fig. 7A). The genotype x allopurinol treatment interaction was not significant for grain yield. Regardless of allopurinol treatment, the two hybrids ('TA4152-37/Alsen' and 'TA4152-19/Alsen') produced significantly lower mean grain yields than their parents. Also, with the exception of 'TA4152-19', allopurinol-treated plants produced significantly lower grain yields than the corresponding non-treated control plants (Fig. 7A). As with plant height, there was no significant difference in grain yield due to allopurinoltreatments.

Second Greenhouse Experiment

There was a significant genotype effect for grain yield (Table 5; Fig. 7B). The F_1 hybrid plants ('TA4152-37/Alsen') produced significantly lower grain yields when compared with both parents. Allopurinol treatment effects and the allopurinol x genotype interaction effect was not significant for grain yield.

Effect of Temperature in Hybrid Necrosis

In both growth chamber studies, there was a significant effect of temperature (Table 6), date and a significant temperature x date interaction for tissue necrosis (Table 6). F_1 plants developed necrosis when grown at both $15^{O}C$ and $23^{O}C$. Significant

differences over time in the development of necrotic tissue were observed 5 d after the first measurements were made (Fig. 8A and 8B). F_1 plants did not develop necrosis while being grown at 30^{o}C in either growth chamber.

In both chambers, temperature had a significant effect on chlorophyll content (Table 7). Genotype and date effects were significant for chlorophyll content (Table 7; Fig. 9A and 9B). Interaction effects of temperature x genotype, and temperature x date also were significant for chlorophyll content (Table 7). Date was not significant for chlorophyll content in either of the TA4152-37 or 'Alsen' genotypes. Hybrid plants grown at 30^{O}C did not develop necrosis, and there was no significant difference in chlorophyll content in leaves measured at 13 and 18 d post emergence as determined from samples taken from the third plant leaf.

The F_1 plants grown at 15^{O}C and 23^{O}C had lower levels of chlorophyll as necrosis progressed, and significantly lower levels were recordedat the second chlorophyll measurement.

Discussion

Plant ROS are involved in many plant responses to biotic and abiotic stresses (Apel and Hirt, 2004, Bailey-Serres and Mittler, 2006). The production of H_2O_2 often triggers the hypersensitive response and cell death in the presence of pathogens or pests (Levine et al., 1994; Lamb and Dixon, 1997). Plants expressing hybrid necrosis have

reportedly produced higher amounts of O_2^- and H_2O_2 before the onset and the progression of tissue necrosis (Khanna-Chopra et al., 1998; Sharma et al., 2003); however results of the present experiments suggest that necrotic tissue development in wheat can occur in the absence of high levels of H_2O_2. In fact, the generation of H_2O_2 decreased as tissue necrosis increased in the hybrid plant leaves sampled in the present experiments. This suggests that wheat likely does not rely exclusively on the production and accumulation of H_2O_2 to activate programmed cell death during the expression of hybrid necrosis. There are documented cases of plant cell death in the absence of an increase in ROS. For example, in zinnia (*Zinnia elegans*) when cultured cells where examined, programmed cell death of tracheary elements did not involve the excess production of ROS (Fukuda, 2000). Also, in an interspecific tobacco hybrid, even the inhibition of O_2^- and H_2O_2 did not halt cell death, and cell death in this case was initiated by an oxidative burst (Mino et al., 2002). In the absence of a ROS scavenging system, excess production of ROS can lead to oxidative plant stress (Apel and Hirt, 2004; Tripathy and Oelmuller, 2012). Sharma et al. (2003) noted that there was a lower concentration of glutathione, ascorbate and SOD in the tissue of plants during the progression of necrosis, and the development of necrotic symptoms in hybrid wheat (Sharma et al., 2003). Ascorbate is used by ascorbate peroxidase and glutathione is used by glutathione peroxidase during the conversion of H_2O_2 into H_2O. Superoxide dismutase plays important role in conversion of superoxide (O_2^-) to H_2O_2 (Montalbini, 1992b; Apel and Hirt, 2004). Despite the lower

SOD content, leaf tissue showed the higher H_2O_2 content in necrotic hybrids (Sharma et al., 2003). In another study, even higher antioxidant enzyme activity did not prevent the cell death associated with the higher ROS (Dalal and Khanna-Chopra, 2001).

There could be two explantions for the progressively lower H_2O_2 levels in the leaves examined in the present experiments. First, there was perhaps no additional generation of ROS during the development of necrotic tissue, which would be in contrast to previous reports of increases in the production of ROS during the expression of hybrid necrosis (Khanna-Chopra et al., 1998; Sharma et al., 2003; Mizuno et al., 2010). A second explanation is that SOD activity was insufficient to bring about the conversion of O_2^- to H_2O_2 even if there was enough O_2^- substrate available.

In the present study, allopurinol treatments effectively inhibited XDH and slightly reduced the production of H_2O_2 in only the first leaves sampled during the development of necrosis; whereas, in the second and fifth leaves there was no impact of the treatment. This suggests that XDH is not the only intermediary in the production of ROS. Other studies demonstrated that the use of allopurinol treatments, in resulted in the inhibition of ROS production and an inhibition of tissue necrosis during a hypersensitive plant response to disease pathogens (Montalbini, 1992b; Montalbini and Torre, 1996). However, in another examination of hybrid necrosis, an increase in ROS was accompanied by an increase in a different ROS, and not an increase in H_2O_2 (Sugie et al., 2007). In plant cells, NADPH oxidase (Bolwell et al., 1995; Apel and Hirt, 2004), pH-

dependent peroxidases (Bolwell et al., 1995) oxalate oxidases (Hu et al., 2003), glycolate oxidases (Liu et al., 2010), and amine oxidases (Walters, 2003) have all been implicated in producing ROS. While in the present experiment the inhibition of XDH inhibition did not equate with a parallel reduction in necrotic tissue in the F_1 hybrids, the increase in chlorophyll content from later sampled tissue acquired from allopurinol treated plants may have been indicative of a partial reduction of ROS. However, this was not accompanied by an increase in grain yield.

In general, plants treated with allopurinol in the first greenhouse expriment exhibited reduced plant height lower grain yield compared with the non-treated control plants. This could be the result of a reduction in uric acid production (Zrenner et al., 2006). A reduction in uric acid may have also resulted in a reduction in the production of allantoin, which would have then been unavailable to convert to N for the plant reassimilation (Werner and Witte, 2011; Zrenner et al., 2006) (Fig. 1). The work of Atkins et al. (1988) on soybean treated with allopurinol demonstrated that it inhibited the formation of uric acid from xanthine, and treated plants exhibited stunted growth and reduced N-fixation in plant nodules. The loss of XDH function in mutants of *Arabidopsis* resulted in a reduction in plant growth and fertility, and ultimately led to premature senescence (Nakagawa et al., 2007; Brychkova et al., 2008). The XDH mutants reverted to the wild-type phenotype after they were treated with urate, a downstream metabolite in uric acid pathway (Nakagawa et al., 2007; Brychkova et al.,

2008). The disruption of the typical plant response to several pathogens because of allopurinol treatments was also attributed to the disruption of the production of allantoin (Montalbini, 1992a; Della Torre and Montalbini, 1995; Adam et al., 2000).

Exogenous allopurinol can also accumulate in plant cells, where it is oxidized to oxipurinol. The activation of purine nucleoside phosphorilase can convert the allopurinol to oxipurinol and the accompanying ribonuleosides for each compound (Krenitsky et al., 1967). Similarly, allopurinol ribonucleosides and oxipurinol ribonucleosides can interfere with more typical host response to a pathogen. For example, as was illustrated in the case of tobacco necrosis virus and leaf rust in wheat (Montalbini, 1995, Montalbini and Della Torre, 1996). Less has been observed about the effects of allopurinol and its downstream derivatives on the agronomic performance of the host plant.

As these ribonucleosides are synthesized, purine nucleoside phosphorilase utilizes xanthosine and inosine (Krenitsky et al., 1967). Inosine and xanthosine are the intermediate compounds that follow the IMP and xanthosine monophosphate (XMP) pathways, respectively before finally converging on the xanthine pathway (Zrenner et al., 2006; Watanabe et al., 2014). The utilization of these two intermediate compounds during the synthesis of ribonucleosides by nucleoside phosphorilase might limit these purine metabolites, and consequently result in a reduction in allantoin and impact the availability of N to plants. However, in wheat, Della Torre and Montalbini (1995)

demonstrated that most exogenous allopurinol is used to inhibit XDH, and very littleallopurinol is converted to ribonucleosides.

Drought, stress, prolonged darkness, and senescence, affect the -production of XDH in plants (Pastori and del Rio, 1997; Nakagawa et al., 2007; Watanabe et al., 2010). As the temperatures in the first greenhouse experiment were cooler i.e., 20 $\pm$ 3^{o}C than the other experiments, it may have impacted the production of xanthine dehydrogenase.

The suppression of necrosis when F_1 plants were grown at 30^{o}C suggests that *Ne1* and/or *Ne2* exhibit some differences in expression in response to temperature differences. This is consistent with the results of Dhaliwal et al. (1986) and others who have observed that the expression levels of necrosis are dependent on temperature (Traw and Bergelson, 2010; Alcazar and Parker, 2011; Hua, 2013). Thus, in the present experiments, there may have been a suppression of the expression of one, or both *Ne* genes as a result of the higher temperature. The effect of temperature on the expression of hybrid necrosis has been observed in other crop species. For example, the production of ROS was reduced and lethality prevented in an interspecific tomato hybrid that typically exhibits hybrid necrosis (Mino et al., 2002).

The slower growth of hybrid plants grown at 15^{o}C in the present experiments compared with those grown at 23^{o}C suggests a similar effect. Wheat plants with the *Net2* gene located to chromosome 2D, and grown at 23^{o}C did not develop necrosis. Conversely, there was a significant level of necrosis expressed when the same plants

were grown at 4°C (Mizuno et al., 2011). Alternatively, expression of the *Nec1* allele located to chromosome 7D of wheat seems not to be impacted in the same way, as hybrids with *Nec1* grown at 30°C did not exhibit a reduced level of necrosis (Mizuno et al., 2010).

In summary, results of the present experiements suggest H_2O_2 is not the only ROS, or perhaps not the only ROS responsible for programmed cell death and the hybrid necrosis expressed in wheat. As O_2^- and other forms of ROS were not measured, these experiments can not conclude that ROS is not solely responsible for the cell death observed during the development of hybrid necrosis in wheat. However, results also suggest that XDH is also not likely to be the main source of ROS generation, or cause of programmed cell death in wheat hybrid necrosis. In the present experiments, and unlike is the case for the allopurinol treatment of wheat plants responding to apathogen, the inhibition of XDH by allopurinol did not have an effect on cell death and wheat hybrid necrosis. The reduction in plant growth and grain yield as a result of allopurinol treatments is likely a consequence of the impact of the chemical on purine ring catabolism, which results in less N being reassimilated by treated plants. Similar to the differential response wheat plants have to various pathogens at comparatively lower and higher temparatures, the results of the present experiments demonstrate that expression of *Ne1* and *Ne2* either together, or independently respond according to differences in temperature. This result supports the concept, and the prediction that the expression of

hybrid necrosis in wheat is a type of auto-immune response that invokes either the same, or similar genes that are often invoked in host-pathogen, or host-pest hypersensitive responses.

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Table 1. ANOVA results for effects of stage of necrosis in F1 plants and allopurinol use in hybrid necrosis of wheat.

Factors	df	*F* value	*P* value
Date	1, 72	314.40	<0.0001
Allopurinol use	2, 72	0.30	0.745
Date X Allopurinol use	2, 72	0.99	0.377

Table 2. ANOVA results for effects of wheat genotypes, allopurinol use and stages of necrosis in necrotic F_1 plants to the H_2O_2 generation in leaves.

Experiments	Factors	df	*F* value	*P* value
2nd greenhouse study				
5th leaf				
	Date	1, 132	5.60	0.019
	Genotype	2, 132	282.70	<0.0001
	Allopurinol use	1, 132	0.87	0.353
	Date X Genotype	2, 132	42.48	<0.0001
	Date X Allopurinol use	1, 132	0.11	0.735
	Genotype X Allopurinol use	2, 132	0.74	0.478
	Date X Genotype X Allopurinol use	2, 132	0.64	0.53
3rd greenhouse study				
Ist leaf				
	Date	1, 132	638.01	<0.0001
	Genotype	2, 132	46.82	<0.0001
	Allopurinol use	1, 132	18.79	<0.0001
	Date X Genotype	2, 132	45.67	<0.0001
	Date X Allopurinol use	1, 132	0.90	0.345
	Genotype X Allopurinol use	2, 132	0.01	0.9903
2nd leaf				
	Genotype	2, 66	138.83	<0.0001
	Allopurinol use	1, 66	3.32	0.073
	Genotype X Allopurinol use	2, 66	0.75	0.47

Table 3. ANOVA results for effects of wheat genotypes, allopurinol use and stages of necrosis in necrotic F1 plants to the chlorophyll content in leaves.

Experiments	Factors	df	*F* value	*P* value
2nd greenhouse study				
3rd leaf				
	Date	1, 198	126.73	<0.0001
	Genotype	2, 198	1149.27	<0.0001
	Allopurinol use	2, 198	0.71	0.495
	Date X Genotype	2, 198	181.11	<0.0001
	Date X Allopurinol use	2, 198	0.46	0.63
	Genotype X Allopurinol use	4, 198	2.09	0.083
	Date X Genotype X Allopurinol use	4, 198	0.57	0.68
8th leaf				
	Date	1, 222	726.14	<0.0001
	Genotype	2, 222	1312.15	<0.0001
	Allopurinol use	2, 222	11.31	<0.0001
	Date X Genotype	2, 222	435.71	<0.0001
	Date X Allopurinol use	2, 222	0.91	0.40
	Genotype X Allopurinol use	4, 222	2.28	0.06
	Date X Genotype X Allopurinol use	4, 222	0.70	0.589
3rdgreenhouse study				
3rd leaf	Date	1, 147	242.74	<0.0001
	Genotype	2, 147	1134.11	<0.0001
	Allopurinol use	1, 147	0.05	0.821
	Date X Genotype	2, 147	141.71	<0.0001
	Date X Allopurinol use	1, 147	0.26	0.612
	Genotype X Allopurinol use	2, 147	0.47	0.628
	Date X Genotype X Allopurinol use	2, 147	0.02	0.981

Table 4. ANOVA results for effect of allopurinol use in plant height of wheat genotypes.

Experiments	Factors	df	*F* value	*P* value
Ist greenhouse study				
	Genotype	4, 79	86.23	<0.0001
	Allopurinol use	3, 79	8.29	<0.0001
	Genotype X Allopurinol use	12, 79	1.10	0.0371
2nd greenhouse study				
	Genotype	2, 78	674.21	<0.0001
	Allopurinol use	2, 78	0.01	0.992
	Genotype X Allopurinol use	4, 78	0.26	0.905

Table 5. ANOVA results for effect of allopurinol use in grain yield of wheat genotypes.

Experiments	Factors	df	*F* value	*P* value
Ist greenhouse study				
	Genotype	4, 73	94.30	<0.0001
	Allopurinol use	3, 73	3.77	0.014
	Genotype X Allopurinol use	12, 73	0.59	0.845
2nd greenhouse study				
	Genotype	2, 74	197.34	<0.0001
	Allopurinol use	2, 74	0.52	0.596
	Genotype X Allopurinol use	4, 74	0.33	0.853

Table 6. ANOVA results for effect of temperature in necrosis development in necrosis exhibiting F_1 plants in wheat

Experiments	Factors	df	*F* value	*P* value
Conviron growth chamber study 1				
	Date	1, 56	82.14	<0.0001
	Temperature	2, 56	49.33	<0.0001
	Date X Temperature	2, 56	24.37	<0.0001
Perceival Scientific Growth chamber study 2				
	Date	1, 62	97.27	<0.0001
	Temperature	2, 62	70.57	<0.0001
	Date X Temperature	2, 62	26.65	<0.0001

Table 7. ANOVA results for effects of wheat genotypes, temperature and stages of necrosis in F1 plants to the chlorophyll content in leaves.

	Factors	df	*F* value	*P* value
Conviron growth chamber study 1				
	Date	1, 164	24.96	<0.0001
	Genotype	2, 164	710.23	<0.0001
	Temperature	2, 164	181.14	<0.0001
	Date X Genotype	2, 164	44.22	<0.0001
	Date X Temperature	2, 164	6.99	0.001
	Genotype X Temperature	4, 164	32.25	<0.0001
	Date X Genotype X Temperature	4, 164	6.17	0.0001
Perceival Scientific growth chamber study 2				
	Date	1, 158	40.77	<0.0001
	Genotype	2, 158	476.51	<0.0001
	Temperature	2, 158	19.58	<0.0001
	Date X Genotype	2, 158	27.35	<0.0001
	Date X Temperature	2, 158	7.00	0.016
	Genotype X Temperature	4, 158	24.08	<0.0001
	Date X Genotype X Temperature	4, 158	6.74	<0.0001

Fig. Legends

Fig. 3. Necrosis progression in fifth leaves of F_1 plants (TA4152-37/Alsen) treated with different concentrations of allopurinol in greenhouse.

Fig. 4. H_2O_2 accumulation in, A) first leaves, B) second leaves, and C) fifth leaves of three wheat genotypes treated with or without allopurinol condition. H_2O_2 measurement was based on the different stages of necrosis in F_1 plants (TA4152-37/Alsen). Error bars are standard errors of means. Bars with different letters are significantly different within a same genotype (P <0.05, least significant difference).

Fig. 5. Chlorophyll content in, A) third leaves, B) eighth leaves, and C) third leaves of three wheat genotypes treated with different concentrations of allopurinol. A and B are from first and C is from second greenhouse experiment study in spring wheat breeding greenhouse. Error bars are standard errors of means. Bars with different letters are significantly different within a same genotype (P <0.05, least significant difference).

Fig. 6. Effect of allopurinol in plant height of wheat genotypes tested at two greenhouse experiments, A) greenhouse located at the Seed Technology Laboratory building, and B) spring wheat breeding greenhouse. Error bars are standard errors of means. Bars with different letters are significantly different within a same genotype (P <0.05, least significant difference).

Fig. 7. Effect of allopurinol on grain yield of wheat genotypes tested at two greenhouse experiments, A) greenhouse located at Seed Technology Laboratory building, and B) spring wheat breeding greenhouse. Error bars are standard errors of means. Bars with different letters are significantly different within a same genotype (P <0.05, least significant difference).

Fig. 8. Necrosis progression in third leaves of F_1 plants (TA4152-37/Alsen) grown at three different temperatures at A) Conviron, and B) Percival Scientific growth chambers. F_1 plants did not develop necrosis at 30ºC and so they did not appear in bar graph.. Bars with an asterisk (*) sign indicates significantly different (P <0.05, least significant difference) within the same temperature range.

Fig. 9. Chlorophyll content in third leaves of three wheat genotypes grown at three different temperatures at A) Conviron, and B) Percival Scientific, growth chambers. Bars with different letters are significantly different within a same genotype (P <0.05, least significant difference).

Figures

Fig. 3.

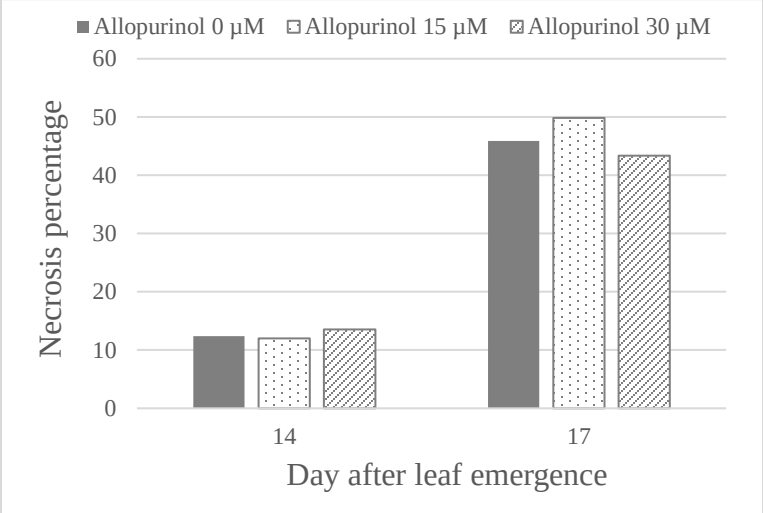


Fig. 4.

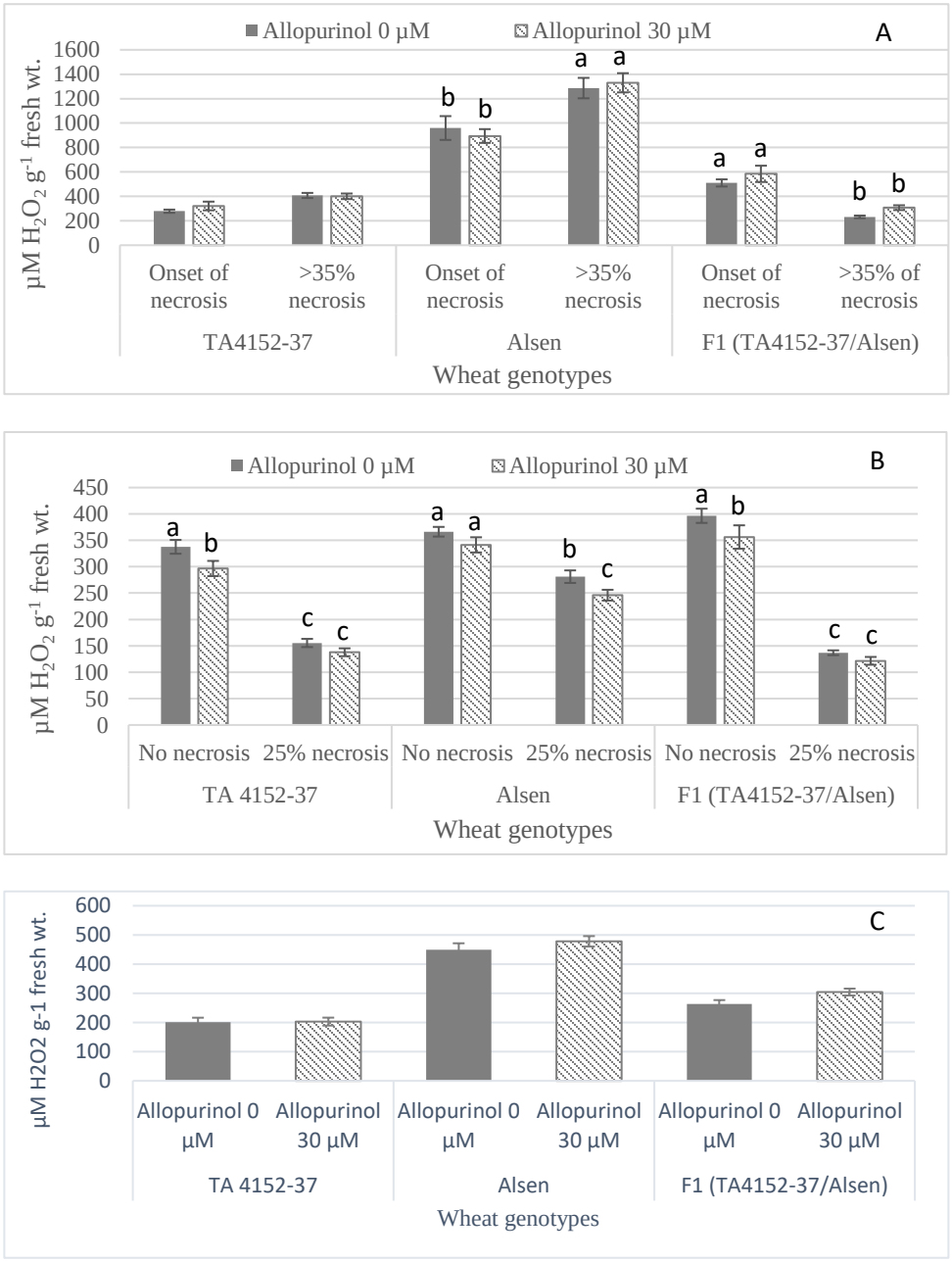

Allopurinol 0 µM
Allopurinol 30 µM
A
µM H2O2 g-1 fresh wt.
1600
1400
1200
1000
800
600
400
200
0
a a b b a a b b
Onset of necrosis
>35% necrosis
Onset of necrosis
>35% necrosis
Onset of necrosis
>35% of necrosis
TA4152-37
Alsen
F1 (TA4152-37/Alsen)
Wheat genotypes
Allopurinol 0 µM
Allopurinol 30 µM
B
µM H2O2 g-1 fresh wt.
450
400
350
300
250
200
150
100
50
0
a b c c a a b c a b c c
No necrosis
25% necrosis
No necrosis
25% necrosis
No necrosis
25% necrosis
TA 4152-37
Alsen
F1 (TA4152-37/Alsen)
Wheat genotypes
C
µM H2O2 g-1 fresh wt.
600
500
400
300
200
100
0
Allopurinol 0 µM
Allopurinol 30 µM
Allopurinol 0 µM
Allopurinol 30 µM
Allopurinol 0 µM
Allopurinol 30 µM
TA 4152-37
Alsen
F1 (TA4152-37/Alsen)
Wheat genotypes

Fig. 5.

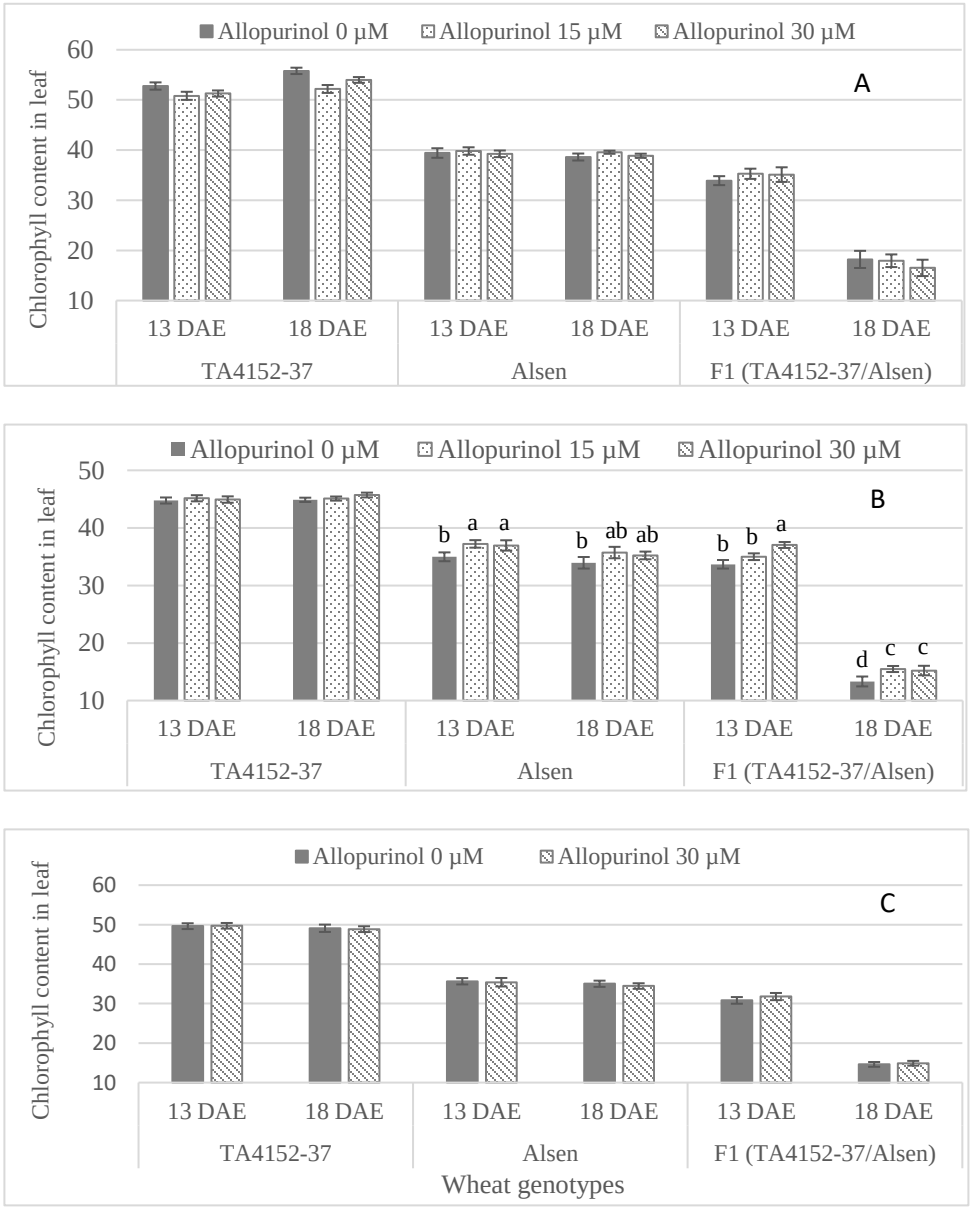
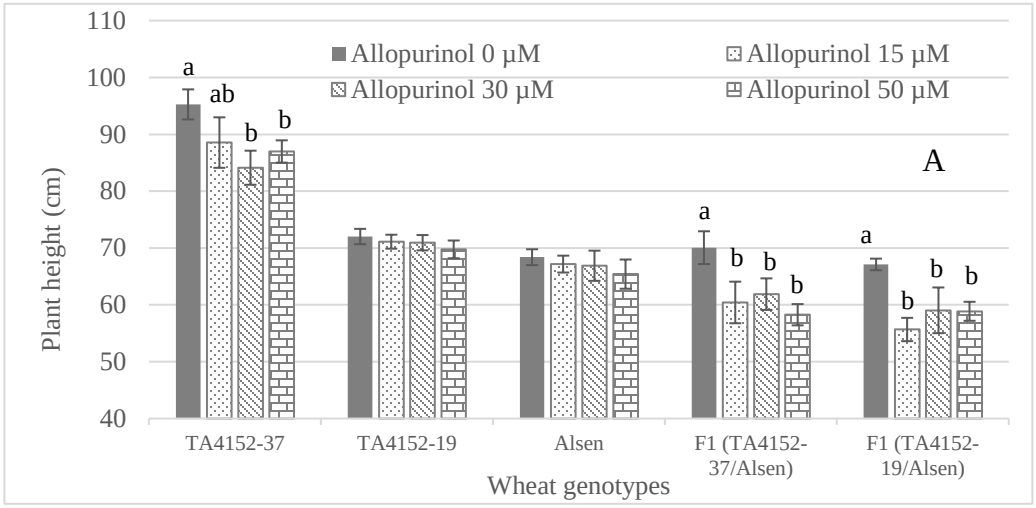

Allopurinol 0 µM
Allopurinol 15 µM
Allopurinol 30 µM
Chlorophyll content in leaf
60
50
40
30
20
10
A
13 DAE
18 DAE
13 DAE
18 DAE
13 DAE
18 DAE
TA4152-37
Alsen
F1 (TA4152-37/Alsen)
Allopurinol 0 µM
Allopurinol 15 µM
Allopurinol 30 µM
Chlorophyll content in leaf
50
40
30
20
10
B
b
a
a
b
ab
ab
b
b
a
d
c
c
13 DAE
18 DAE
13 DAE
18 DAE
13 DAE
18 DAE
TA4152-37
Alsen
F1 (TA4152-37/Alsen)
Allopurinol 0 µM
Allopurinol 30 µM
Chlorophyll content in leaf
60
50
40
30
20
10
C
13 DAE
18 DAE
13 DAE
18 DAE
13 DAE
18 DAE
TA4152-37
Alsen
F1 (TA4152-37/Alsen)
Wheat genotypes

Fig. 6.


110
100
90
80
70
60
50
40
Plant height (cm)
Allopurinol 0 µM
Allopurinol 15 µM
Allopurinol 30 µM
Allopurinol 50 µM
a
ab
b
b
a
b
b
b
a
b
b
b
A
TA4152-37
TA4152-19
Alsen
F1 (TA4152-37/Alsen)
F1 (TA4152-19/Alsen)
Wheat genotypes

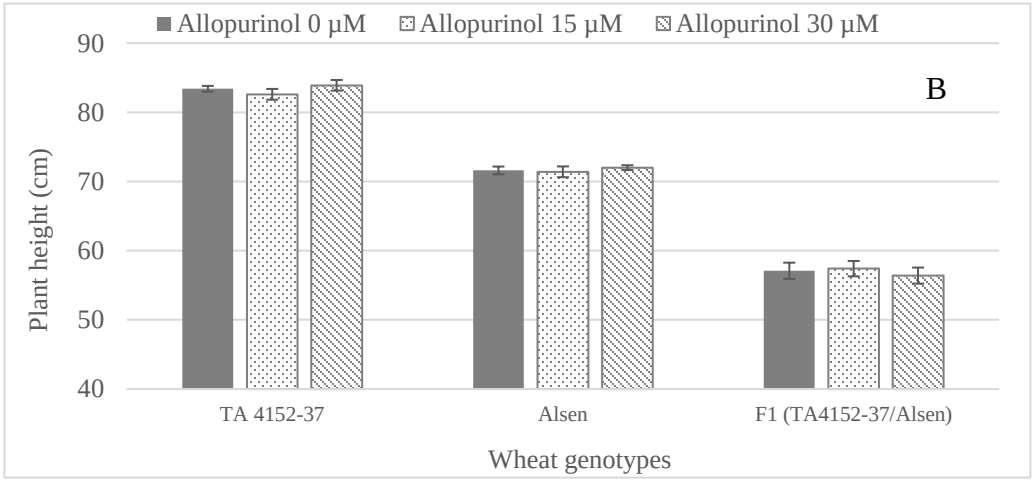
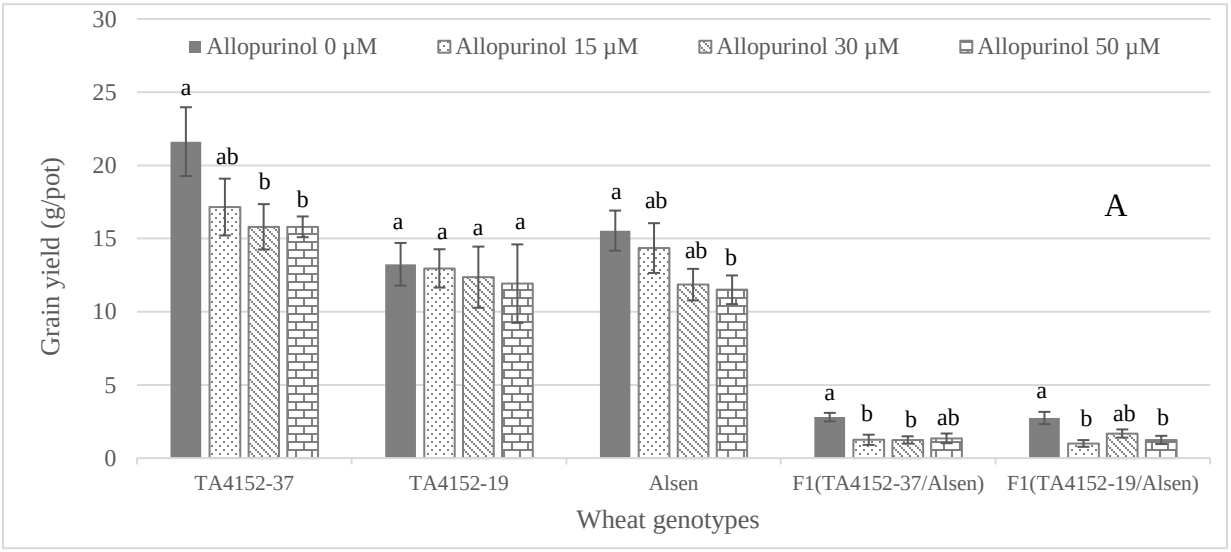

Allopurinol 0 µM
Allopurinol 15 µM
Allopurinol 30 µM
90
80
70
60
50
40
Plant height (cm)
B
TA 4152-37
Alsen
F1 (TA4152-37/Alsen)
Wheat genotypes

Fig. 7.


Allopurinol 0 µM
Allopurinol 15 µM
Allopurinol 30 µM
Allopurinol 50 µM
Grain yield (g/pot)
30
25
20
15
10
5
0
a
ab
b
b
a
a
a
a
a
ab
ab
b
a
b
b
ab
a
b
ab
b
A
TA4152-37
TA4152-19
Alsen
F1(TA4152-37/Alsen)
F1(TA4152-19/Alsen)
Wheat genotypes

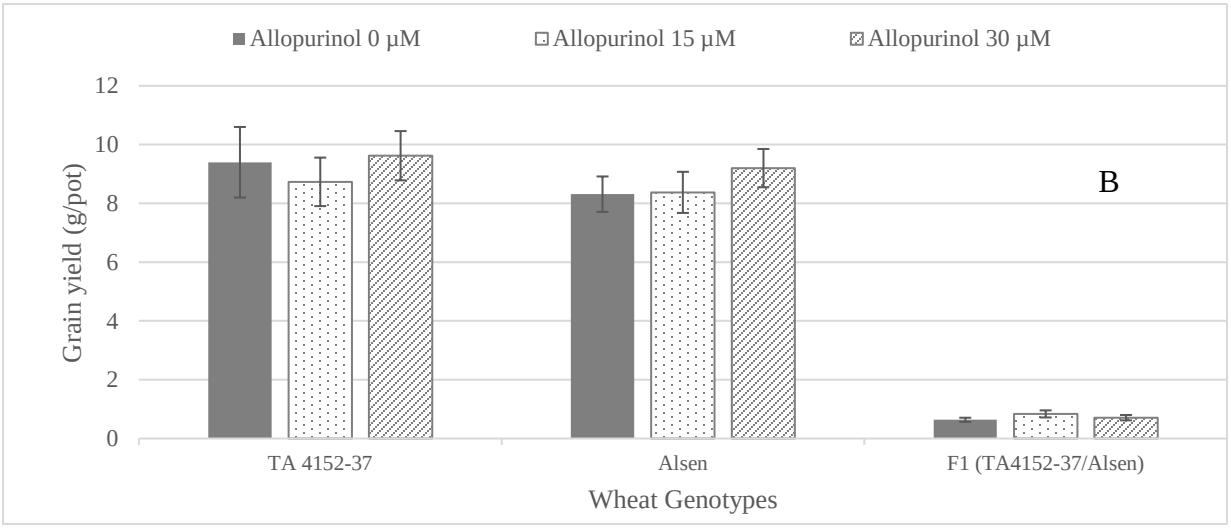

Allopurinol 0 µM
Allopurinol 15 µM
Allopurinol 30 µM
Grain yield (g/pot)
12
10
8
6
4
2
0
B
TA 4152-37
Alsen
F1 (TA4152-37/Alsen)
Wheat Genotypes

Fig. 8.

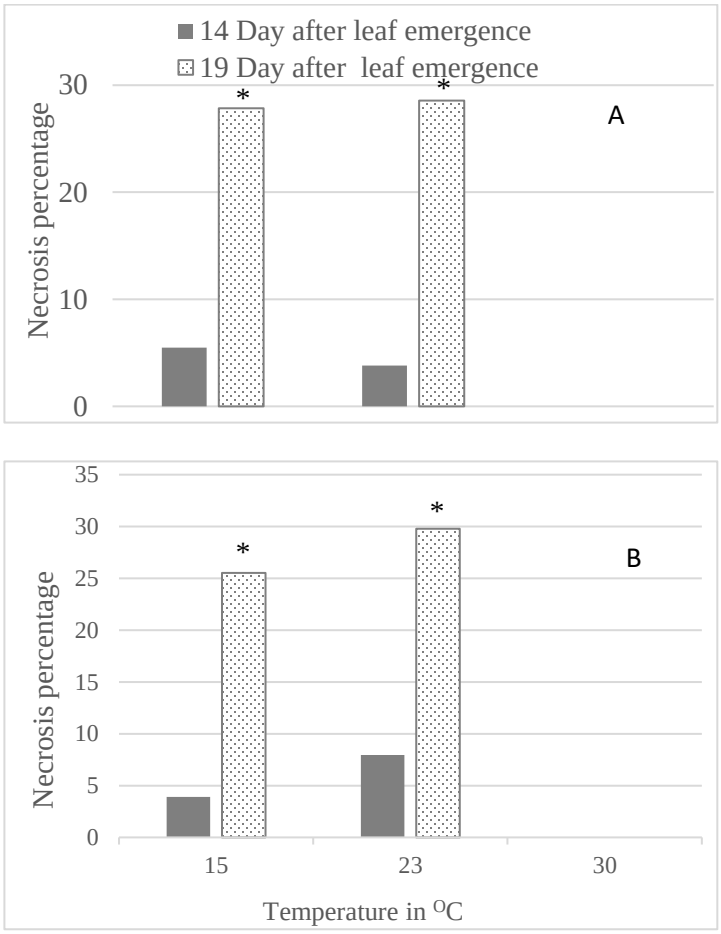
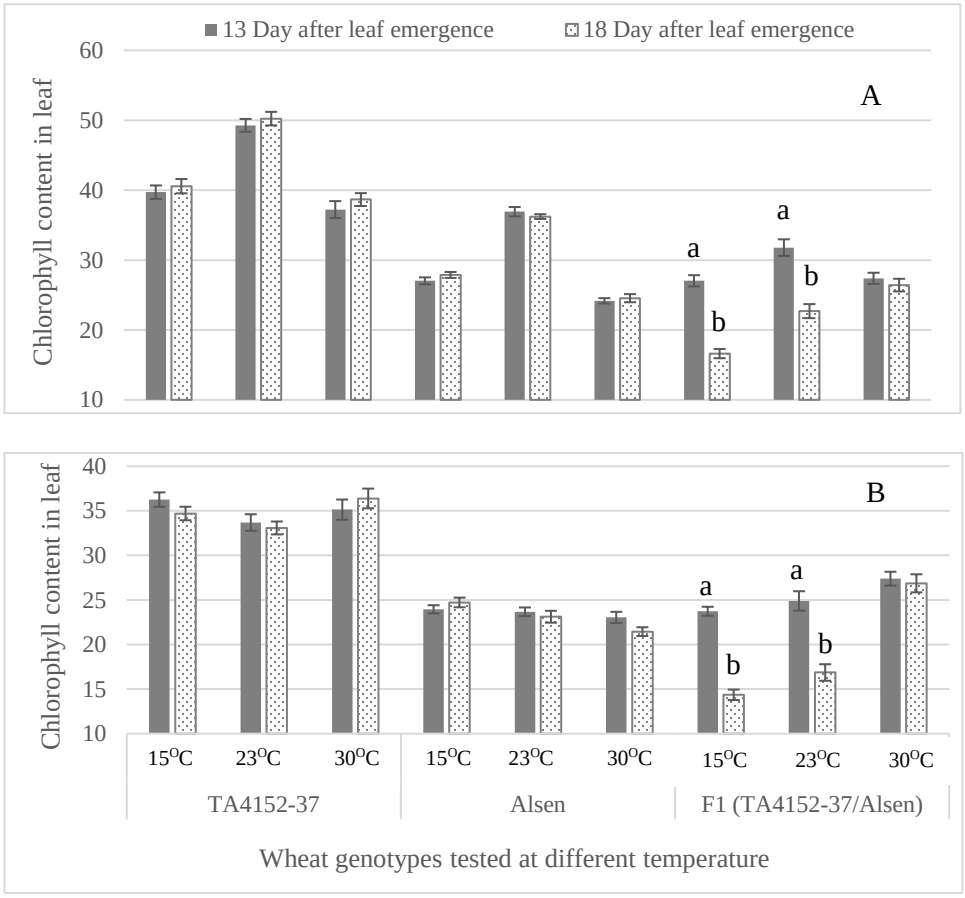

14 Day after leaf emergence
19 Day after leaf emergence
Necrosis percentage
30
20
10
0
*
*
A
35
30
25
20
15
10
5
0
*
*
B
15
23
30
Temperature in OC

Fig. 9.


13 Day after leaf emergence
18 Day after leaf emergence
Chlorophyll content in leaf
60
50
40
30
20
10
A
a
b
a
b
Chlorophyll content in leaf
40
35
30
25
20
15
10
B
a
b
a
b
15°C
23°C
30°C
15°C
23°C
30°C
15°C
23°C
30°C
TA4152-37
Alsen
F1 (TA4152-37/Alsen)
Wheat genotypes tested at different temperature

CHAPTER 3: Flanking SSR Markers for Alleles Involved in the Necrosis of Hybrids between Hexaploid Bread Wheat (*Triticum aestivum* L.) and Synthetic Hexaploid Wheat

Abstract

Hybrid necrosis in wheat (*Triticum aestivum* L.) results from the interaction of two dominant complementary genes, *Ne1* and *Ne2*, located to chromosomes 5B and 2B, respectively. We examined allelic interaction effects of necrosis alleles using simple sequence repeat (SSR) markers in F_2 populations derived from crossing the cultivar 'Alsen' with a synthetic hexaploid 'TA4152-37'. The SSR marker *Xbarc7* was linked at a distance of 3 cM to the quantitative trait loci (QTL) located on chromosome 2B, and *Xgwm639* was 11 cM from the 5B QTL. A significant additive by additive epistatic interaction was detected between *Ne1* and *Ne2* QTL, and results suggest that 'Alsen' possesses a moderate necrosis allele, $Ne2^m$; whereas, 'TA4152-37' possesses a moderate necrosis allele, $Ne1^m$. The $Ne2^m$ allele had a stronger effect than $Ne1^m$, and a total of 94.6% of the phenotypic variance was explained by these genes and their interactions. This demonstrates the strong phenotypic effect when moderate necrosis alleles are being expressed in wheat hybrids, and it emphasizes the need for breeders to accurately predict and identify hybrids that will produce necrosis symptoms.

Introduction

Hybrid necrosis in wheat (*T. aestivum*) is a condition resulting in the premature necrosis of green tissue and the death of leaves, or entire plants (Tsunewaki, 1960; Hermsen, 1963a; Tomar et al., 1991; Tomar and Singh, 1998). It is the result of the complementary interaction of two dominant genes, *Ne1* and *Ne2*, which are located to chromosomes 5B and 2B, respectively (Tsunewaki, 1960; Zeven, 1972; Nishikawa et al., 1974; Chu et al. 2006). Necrosis induced by the *Ne1* and *Ne2* genes is also referred to as a type I hybrid necrosis (Tsunewaki, 1960, 1970; Chu et al., 2006; Mizuno et al., 2010). Hybrid necrosis can result in a "bottleneck" to the breeding process because it may prevent gene transfer between otherwise desirable parents, or restrict the transfer of desirable genes to cultivars from related species.

Wheat genotypes that carry the *Ne1* or *Ne2* genes exist across a wide geographic distribution (Pukhalskiy et al., 2000; Singh et al. 2000). Approximately, 55% of wheat genotypes from North America, 65% from India and 6.7% from Africa carry the *Ne2* gene; whereas, 7.5% of wheat from North America, 7.7% from India, and 47.9% from Africa possess the *Ne1* gene (Pukhalskiy et al., 2000; Vikas et al. 2013). The *Ne2* gene on chromosome 2B is tightly linked with the *Lr13* gene, which confers resistance to leaf rust (*Puccinia triticina* Eriks) (McIntosh et al., 1995). The work of Zhang et al. (2016) advocated that Lr13 and *Ne2* could in fact be the same genes. Moreover, *Lr13* is tightly linked with *Lr23* (McIntosh et al. 1995). These two leaf rust resistance genes have been

widely employed in wheat breeding (Vikas et al., 2013), and the short arm of chromosome 2B carries the stem rust (*Puccinia graminis* f. sp. *tritici*) resistance genes, *Sr36* and *Sr40,* both of which are also currently being extensively utilized by wheat breeders (Vikas et al., 2013). Breeding for rust resistance may have inadvertently resulted in an increase in the frequency of the *Ne2* gene within some germplasm pools (Oelke and Kolmer, 2005, Bomblies and Weigel 2007).

Hybrid necrosis is depicted as an autoimmune plant response in which genes related to defense mechanisms are thought to play a significant role (Bomblies and Weigel, 2007). In *Arabidopsis*, lettuce, and tomato, the pleotropic effects of several disease resistance genes were observed to trigger hybrid necrotic responses (Krüger et al., 2002; Bomblies et al., 2007; Jeuken et al., 2009; Alcázar et al., 2010). In wheat, disease response genes were found to be significantly up-regulated among both intraspecific hybrids (Zhang et al., 2014) and interspecific hybrids between tetraploid wheat and *Aegilops tauschii* (Mizuno et al., 2010). Wheat plants that exhibit hybrid necrosis also produce higher reactive oxygen species, which have been associated with programmed cell death (Khanna-Chopra et al., 1998; Sharma et al. 2003). Programmed cell death is integral to the hypersensitive response, which is a classical plant response tohost/pathogen and host/pest gene-for-gene interactions (Mur et al., 2008).

The Dobzhansky-Muller (DM) model (Dobzhansky and Dobzhansky, 1937; Muller, 1942) has been proposed as an explanation for the incompatibility that results in

hybrid necrosis. It supposes that as ancestral populations diverged and evolved, divergent lines underwent independent genomic substitutions. Although such substitutions do not negatively impact individual lines, when such divergent lines hybridize, or are hybridized, the result is genetic incompatibility evidenced by tissue necrosis (Dobzhansky and Dobzhansky, 1937; Muller, 1942; Bomblies and Weigel, 2007). Further, this incompatibility serves as a post-zygotic reproductive barrier (Hermsen, 1967; Orr, 1996; Bomblies et al., 2007). The same model can also explain the reduction of fitness in hybrids because of the interaction of epistatic loci when divergent lines are combined. Also, in polyploid species with multiple genomes, genetic incompatibility and hybrid necrosis can be even greater when compared with diploid species (Tikhenko et al., 2008; Hatano et al., 2012).

Although hybrid necrosis in wheat is caused by the complementary interaction of the *Ne1* and *Ne2* genes, varying levels of necrosis have been reported. The work of Hermsen (1963a, 1963b), Zeven (1972), and Singh et al. (1992) suggests that there are three alleles at the *Ne1* locus (i.e., $Ne1^{w}$-weak, $Ne1^{m}$–moderate, and $Ne1^{s}$-strong alleles) and five alleles at the *Ne2* locus (i.e., $Ne2^{w}$-weak, $Ne2^{mw}$-moderate to weak, $Ne2^{m}$-moderate, $Ne2^{ms}$-moderate to strong, and $Ne2^{s}$-strong alleles,) and combinations of weak and strong alleles at the *Ne1* and *Ne2* loci may account for the different levels of necrosis observed among F_1 hybrids (Hermsen 1963a). Takumi et al. (2013) examined plants combining a strong necrosis allele at *Ne2* with a weak allele at *Ne1*, and the result was

leaf necrosis at heading, but there were no obvious reductions in seed fertility and seed weight.

The hard red spring wheat cultivar, ‘Alsen’ (Frohberg et al., 2006) possesses the *Ne2* gene, and the *Ne1* gene has been identified in several synthetic hexaploid wheat lines developed by the International Maize and Wheat Improvement Center (CIMMYT) (Chu et al., 2006). Hybrids between Alsen and the synthetic hexaploid wheat line TA4152-37 exhibited moderate levels of necrosis (Chu et al., 2006). This parental combination can be used to test some of the proposed models for hybrid necrosis and to determine which of the weak, moderate, or strong necrosis alleles are responsible for the phenotype. The objectives of this research were to further characterize the necrosis alleles involved in determining the phenotype of hybrids involving ‘Alsen’ and the synthetic hexaploid wheat, ‘TA4152-37’, and to possibly identify molecular markers more tightly linked to the *Ne* alleles involved in the necrotic response.

Materials and Methods

Plant Material

The hard red spring wheat cultivar, ‘Alsen’ and synthetic hexaploid wheat line, ‘TA4152-37’ were used to develop a mapping population. ‘Alsen’ has been widely grown throughout the Northern Plains of the USA, primarily because it expresses a high level of resistance to Fusarium head blight (FHB) (Frohberg et al., 2006). ‘Alsen’ has the

pedigree ND674//ND2710/ND688 (Frohberg et al. 2006), and it derives its FHB resistance from ND2710, which was also developed by the NDAES, and it has the pedigree ‘Sumai 3’/’Wheaton’//’Grandin’ (Frohberg et al., 2004). The synthetic hexaploid line ‘TA4152-37’ has the pedigree, 68.111/RGB-U//’Ward’/3/FGO/4/RABI/5/*Ae. tauschii* (878) (Xu et al., 2004; Chu et al., 2006).

The F_1 hybrids for this study were produced by using ‘Alsen’ as the male parent and ‘TA4152-37’ as the female. The harvested F_2 seeds from hybrids and the parents were sown into plastic pots measuring 12 cm x 11 cm and grown in a greenhouse at South Dakota State University. Pots were filled withSunshine Mix 1 (Sun Gro Horticulture Canada Ltd., Seba Beach, AB, Canada), and the greenhouse temperature was maintained at 25 ± 3^{o}C with a 16 h day length maintained with supplemental lighting.

Phenotype Evaluation

Soon after germination, F_2 plants were visually evaluated for development of necrosis symptoms and rated for the severity of tissue necrosis. Severity was rated on a 0 to 6 scale, which was based on the rating scale employed by Hermsen (1963a; Table 8). Plants appearing to express no necrotic symptoms were rated as 0, while plants expressing a low level of necrosis were rated as 1. Plants exhibiting the most severe necrotic symptomswere rated as 6 (Table 8).

Genotyping

Leaf tissue for DNA extraction was collected by excising leaf sections from plants 14 to 28 d after being sown to pots. Samples were immediately flash-frozen in liquid N and stored at -80^{o}C until extraction. Genomic DNA was isolated according to a modified phenol-chloroform extraction protocol (Karakousis and Langridge, 2003). The quality of DNA for all samples was examined by running extractions on 1% agarose gels. A NanoDrop 1000 spectrophotometer was used to determine DNA concentrations so that samples could be diluted to 50 ng/µl for use in the marker analyses. Sixty-six simple sequence repeat (SSR) markers to chromosome 2B, and 58 to chromosome 5B were selected from a wheat consensus map (Somers et al., 2004). These were used to screen for polymorphisms among parents and within the F_2 population. Primer sequences were synthesized according to the GrainGenes database (http://wheat.pw.usda.gov), and marker amplification was performed using M13 tailing according to Somers et al. (2004). After amplification, 3 µl of each PCR product was mixed with 9 µl of a solution containing GeneScan^{TM}–500 LIZ® and Hi-Di^{TM} Formamide combined at a ratio of 1:60. Resultant mixtures were denatured for 5 min at 94^{o}C and placed on ice. Amplified PCR products were separated using an ABI 3130x1 Genetic Analyzer (Foster City, CA, USA) and analyses of the results were performed using GeneMapper® software v3.7 (Foster City, CA, USA).

Statistical Analysis and QTL Mapping

To test whether two complementary genes were responsible for necrotic symptoms, a Chi-square test was performed for the segregation of necrosis rating against an expected 9 necrotic : 7 non-necrotic ratio in the F_2 population. For mapping, marker positions, linkage groups were assigned according to the consensus map of Somers et al. (2004). Composite interval mapping was performed for QTL analysis using Windows QTL Cartographer software (ver 2.5) (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm) (Wang et al. 2011). The phenotypic variation explained by QTL and additive effects was estimated, and multiple interval mapping was carried out to evaluate epistatic interactions between the QTL. One-way and two-way analyses of variance were performed using the PROC GLM procedure in SAS (v 9.2; SAS Institute Inc., Cary, NC) for SSR markers found to be associated with hybrid necrosis.

Results

QTL Analysis of Necrosis

All F_1 hybrids produced for this study developed necrosis and exhibited limited tillering. They also produced few spikes, and those spikes generally produced shriveled grain. The first symptoms of necrosis in all F_1 plants were observed at the tip of the first leaf when the second leaf was fully expanded. Out of the 198 F_2 plants derived from these necrotic hybrids, 108 developed necrotic symptoms while 90 showed no outward

symptoms of tissue necrosis. A Chi-square test for the 9:7 ratio was significant (Fig. 10), which is indicative of hybrid necrosis in these hybrids being determined by two complementary *Ne* genes. The frequency of necrosis ratings among F_2 plants is presented in Fig. 10. When necrotic tissue became visually evident, the first symptoms appeared as necrosis affecting the tips of the lowest, or oldest leaves when plants were at the one-to-three leaf growth stage. The majority of plants that developed symptoms died before spikes and seeds were produced (60.2% of necrotic plants with scores of 3–6, Fig. 10), and many plants that did produce spikes were sterile (approx. 25.9% of necrotic plants).

Screening of the 66 SSR markers for chromosome 2B revealed that 14 were polymorphic among the parents. The *Xbarc7* marker, mapped to a distance of 3 cM and was most closely linked to the QTL for hybrid necrosis on chromosome 2B (Fig. 11), while *Xbarc13* was linked to this same QTL at a distance of 4 cM. The chromosome 2B QTL was flanked by *Xbarc7* and *Xwmc344* (Fig. 11). A total of 8 SSR markers were found to be polymorphic to the QTL located to chromosome 5B, and *Xgwm639* was most closely linked at a distance of 11 cM (Fig. 11).

A LOD value of 13.7 was associated with the markers located on chromosome 2B, and 35 % of the total phenotypic variance was explained by these markers. The chromosome 5B markers resulted in a LOD value of 12.1, which explained 29.3 % of the total phenotypic variance. Multiple interval mapping showed that the additive effect of the 2B QTL was opposite to that of the QTL located on chromosome 5B (Table 9),

suggesting that the 2B allele from ‘Alsen’, and the 5B allele from ‘TA4152-37’ were both responsible for the observed necrosis. A significant additive by additive epistatic interaction was detected between the two QTL, and 94.6% of the phenotypic variation was explained by both additive and epistatic interactions. (Table 9).

Effect of QTL on Phenotypes

The SSR markers closely linked to the QTL on both chromosomes demonstrated a significant effect on hybrid necrosis (Table 9 and 10). The Alsen alleles near markers *Xbarc7*, *Xbarc13*, and *Xwmc344* were associated with significant effects in determining the necrotic phenotype. The 288 bp, 159 bp, and 269 bp alleles of these markers were associated with necrosis (Table 10), and the 194 bp allele originating from ‘TA4152-37’ and associated with the *Xgwm639* marker was significant for necrosis (Table 10).

The epistatic interactions between the homozygous necrosis QTL on chromosomes 2B and 5B resulted in the most severe necrosis, *Ne2Ne2* on chromosome 2B, and *Ne1Ne1* on chromosome 5B (Fig. 12). The severity of necrosis resulting from a combination of homozygous ‘Alsen’ alleles of the 2B QTL with the heterozygous 5B QTL alleles was similar to necrosis resulting from a combination with the homozygous necrosis alleles from both 2B and 5B (Fig. 12). Genotypes homozygous for the 2B QTL with heterozygous 5B QTL background (*Ne1ne1Ne2Ne2*) developed more severe necrosis when compared to genotypes carrying the TA4152-37 homozygous alleles of the

5B QTL with the heterozygous 2B QTL background (*Ne1Ne1Ne2ne2*) (Fig. 12). Plants having at least one of the QTL homozygous and other heterozygous (*Ne1ne1Ne2Ne2* or *Ne1Ne1Ne2ne2*) exhibited significantly more severe necrotic symptoms compared with plants with heterozygous alleles at both QTL (*Ne1ne1Ne2ne2*) (Fig. 12).

Discussion

The observed 9:7 ratio of necrotic to healthy lines in the F_2 population is consistent with two dominant, complementary necrosis genes segregating within the population. Significant necrotic effects are due to 'Alsen' alleles at the 2B QTL as well as to 'TA4152-37' alleles at the 5B QTL (Table 10). Significant interactions between the alleles illustrate that these QTL contributed to hybrid necrosis. Additionally, the QTLs located on chromosomes 2B and 5B, correspond with the locations of the *Ne2* and *Ne1* genes, respectively, as reported by Chu et al. (2006). The 2B and 5B QTL and their interactions explained 94.6% of the total phenotypic variation for necrosis. This is higher than any phenotypic effects observed due to the interaction of a strong necrosis genotype in combination with a weak necrosis genotype. For example, Takumi et al. (2013) noted that only 40.9% of the total phenotypic variation was explained as the result of such a combination.

The phenotypes of F_1 hybrids in this study were similar to what was described in the hybrids produced by Chu et al. (2006). The degree of necrosis among F_1 hybrids has

been described in several studies, and is dependent on specific allelic combinations of *Ne1* (weak, moderate, or strong allele) and *Ne2* (weak, moderate to weak, moderate, moderate to strong, or strong allele; Hermsen 1963a, 1963b; Singh et al., 1992; Zeven, 1972). The F_1 plants possessing the heterozygous *Ne1ne1Ne2ne2* genotype exhibited reduced numbers of tillers and smaller spikes that were either sterile, or produced shriveled seeds. This level of necrosis was similar to that exhibited by F_2 plants possessing the same genotype (Fig. 12). Still, a broad range of necrotic responses were observed among the F_2 plants. Plants with one heterozygous allele that were also homozygous for the complementary alleles (*Ne1Ne1Ne2ne2* or *Ne1ne1Ne2Ne2*) exhibited early plant death, and did not produce spikes. By comparison, plants heterozygous at each complementary necrosis gene loci exhibited less severe necrosis, possibly because in the case of a homozygous and heterozygous complementary combinations, there is higher dosage of dominant *Ne* alleles. Plants homozygous for *Ne1* and *Ne2* alleles exhibited the most severe necrosis due to additive by additive epistatic effects, and they died as early as the second leaf stage of growth (Fig. 10 and 12; Table 8). Takumi et al. (2013) noted that there was a higher level of hybrid necrosis expressed due to epistatic interactions between weak necrosis alleles ($Ne1^{w}$) and complementary strong necrosis alleles ($Ne2^{s}$). Necrosis levels of plants of the genotype *Ne1ne1Ne2Ne2* were comparable with those of the *Ne1Ne1Ne2Ne2* genotype; however, they also tended to have exhibited a slightly higher level of hybrid necrosis than plants with the

Ne1Ne1Ne2ne2 genotype (Fig. 12). This implies that although the *Ne2* allele from 'Alsen' seems to result in more severe necrosis when compared with the *Ne1* allele from 'TA4152-37', both alleles may have evolved from the same ancestral loci to induce similar levels of hybrid necrosis. Our results suggest that the 'Alsen' and 'TA4152-37' necrosis genotypes are; *ne1ne1Ne2*^{m}*Ne2*^{m}, and *Ne1*^{m}*Ne1*^{m}*ne2ne2,* respectively. Both designations are consistent with the observation of moderate necrosis in F_1 plants (Hermsen 1963a, Chu et al. 2006), and the proposed genotype for Alsen is also consistent with Chu et al. (2006), and the confirmed linkage of *Lr13* with a moderate necrosis, *Ne2* gene (Singh, 1993; Wamishe and Milus 2004).

The identification of additional molecular markers linked to these necrosis QTL could prove useful for fine-mapping and the eventually cloning of *Ne* genes. Such markers would be useful in evaluating parents for necrosis prior to crossing, and for practicing marker-assisted selection to eliminate the dominant *Ne* alleles from important germplasm pools. In comparison to the results of Chu et al. (2006), our study identified three additional SSR markers (i.e., *Xbarc7, Xbarc13* and *Xwmc344*) that are closely linked with the *Ne2* gene on chromosome 2B. Additionally, *Xgwm639* is closely linked with *Ne1* gene on chromosome 5B. Chu et al. (2006) reported that *Xbarc55* was linked to *Ne2* at a distance of 3.2 cM on the proximal side, while we found that *Xbarc7* was linked at a distance of 3 cM on the distal side. Additionally, the present study determined that *Xbarc7* is more tightly linked to *Ne2* than is the previously identified marker for this

gene, *Xgwm148* (Somers et al., 2004; Chu et al., 2006). Therefore, flanking markers *Xbarc55* and *Xbarc7* are expected to be more effective markers for the *Ne2* gene. We also found that *Xgwm639* resides at a distance of 11 cM from *Ne1* on the distal side.

These results illustrate the strong negative impact on plant phenotype due to the epistatic interactions of dominant, complementary *Ne* alleles, and this effect is even evident in genotypic combinations that involve moderate necrosis alleles. Since many common wheat cultivars and landraces contain either the *Ne1* or *Ne2* genes (Hermsen 1963b; Pukhalskiy et al., 2000, 2009), and the frequency of the *Ne2* gene has likely increased in wheat germplasm pools due to selection for rust resistance (McIntosh et al., 1995; Pukhalskiy et al., 2000; Bomblies and Weigel, 2007), it behooves breeders to be cognizant of the potential to inadvertently produce hybrid necrotic genotypes, and thus undermine the goal of making desirable parental combinations. Additionally, although the synthetic hexaploid wheats are thought to represent a rich pool of diversity for wheat breeders, there is the elevated risk of introgressing the *Ne1* gene from synthetic hexaploid wheat lines when attempting to access this germplasm pool. Synthetics are often produced from hybrids between tetraploid wheat and the diploid, *Ae. tauschii*, and *Ne1* is common in tetraploid species (Hermsen, 1963b; Tsunewaki 1970, 1992).

Along with previously reported molecular markers (Chu et al., 2006; Takumi et al. 2013), those identified in this study may serve a broader breeding objective. They

might be used to help more efficiently and effectively eliminate hybrid necrosis genes from within segregating populations.

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Table 8. Description of scores used to evaluate necrosis severity among F_2 wheat plants derived from crosses of 'TA4152-37' with 'Alsen'.

Necrosis rating	Appearance of first symptoms	Symptoms and growth stages of symptoms
0	----------	No necrosis or stunted growth
1	2–3 leaves	Reduced number of spikes, spikes with small seeds
2	2–3 leaves	Later flowering, reduced number of spikes, seedless spikes
3	2 leaves	Dying at boot stage
4	2 leaves	Dying at 6–8 leaf stage, small leaves
5	1 -2 leaves	Dying at 4 -5 leaf stage, small leaves
6	1 leaf	Dying at 2–3 leaf stage

Table 9. QTL and their additive effects on expression of hybrid necrosis among F_2 wheat plants derived from crosses of 'TA4152-37' with 'Alsen'.

QTL	Type	Position (cM)	Effect	Effect (%)	*P* value
2B	A	50.01	1.55	30.3	<0.0001
5B	A	42.01	-1.74	48.4	<0.0001
2B X 5B	AA		1.35	15.9	0.0006

(A = Additive effect, AA = Additive by Additive effect)

Table 10. Chromosomal locations, allele size and mean necrosis rating of SSR markers associated with hybrid necrosis.

Marker	Marker position (cM)	Allele size (bp)[1]	Mean ± Std. error[2]	*P* value
Xbarc7-2B	47	288	2.92 ± 0.45a	<.0001
		291	0.70 ± 0.32b	
Xbarc13-2B	46	159	2.93 ± 0.43a	<.0001
		162	1.0 ± 0.36b	
Xwmc344-2B	56	269	2.57 ± 0.49a	<.0001
		246	0.30 ± 0.19b	
Xgwm639-5B	53	189	0.44 ± 0.19b	<.0001
		194	2.85 ± 0.47a	

[1] Mean and standard (Std.) error are based on necrosis range from 0 to 6, 0 is for no necrosis, 1 is for the least and 6 is for the highest level of necrosis development.

[2] Mean value with different letters are significantly different within a marker ($P<0.05$, least significant difference).

Fig. Legends

Fig. 10. Frequency of necrosis ratings for F_2 population. Rating of 0 = no necrosis, 1 = least to 6 most necrosis.

Fig. 11. Linkage maps and QTL associated with the necrosis alleles on chromosomes 2B and 5B. Linkage groups and markers position are based on the wheat consensus map (Somers et al. 2004). Genetic distances are represented in cM on the left, and corresponding markers are presented on the right side of each chromosome. Vertical line = LOD threshold score of 2.5.

Fig. 12. Allelic interaction effects of necrosis 2B QTL with necrosis 5B QTL. Error bars are standard errors of means. Error bars with different letters are significantly different ($P<0.05$, least significant difference).

Figures

Fig. 10.

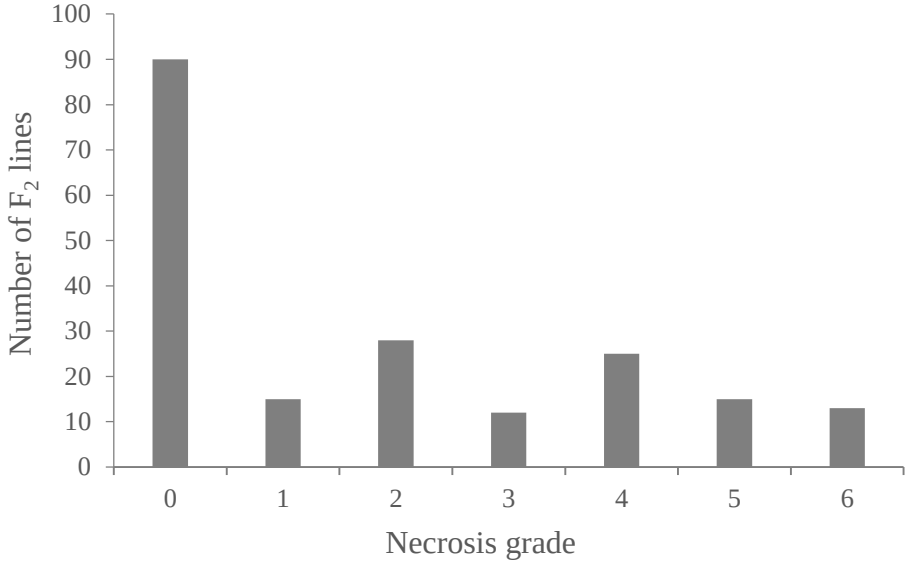


Fig. 11.

Fig. 11.1 Chromosome 2B

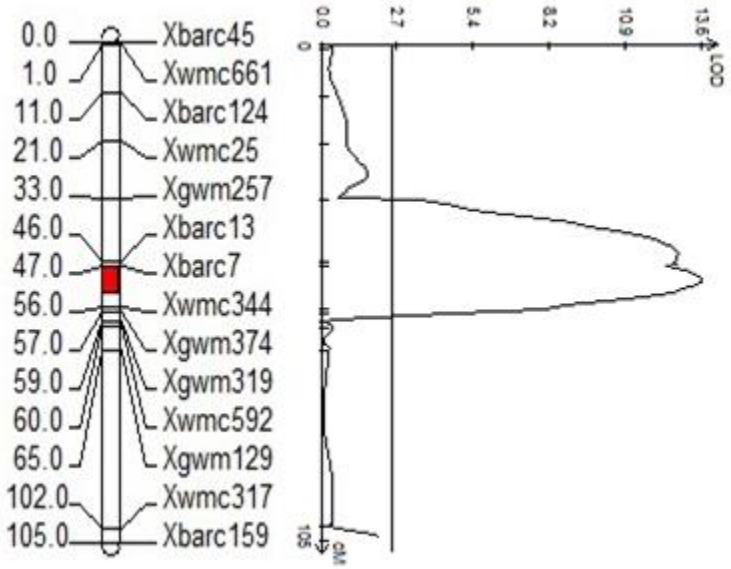


Fig. 11.2 Chromosome 5B

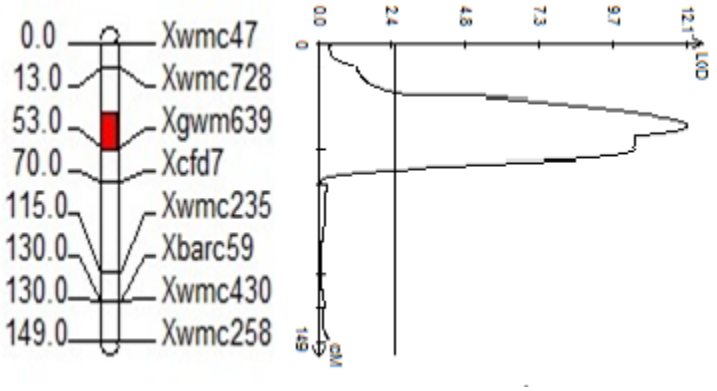
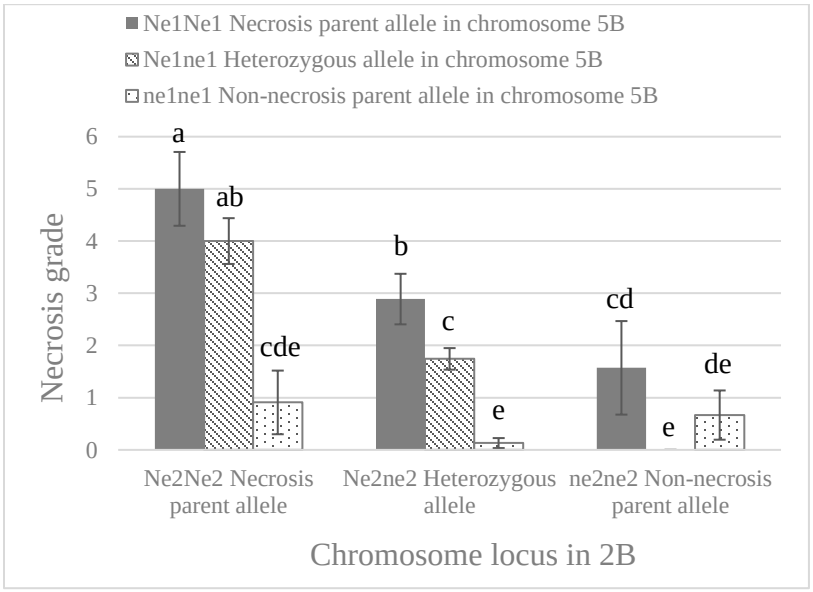


Fig. 12.


Ne1Ne1 Necrosis parent allele in chromosome 5B
Ne1ne1 Heterozygous allele in chromosome 5B
ne1ne1 Non-necrosis parent allele in chromosome 5B
Necrosis grade
0
1
2
3
4
5
6
a
ab
cde
b
c
e
cd
e
de
Ne2Ne2 Necrosis parent allele
Ne2ne2 Heterozygous allele
ne2ne2 Non-necrosis parent allele
Chromosome locus in 2B

CHAPTER 4: RNA-Seq Analysis of Hybrid Necrosis in Wheat (*Triticum aestivum* L.)

Abstract

Hybrid necrosis results from a genetic incompatibility and can be lethal or result in a severe reduction in grain yield and plant productivity. Molecular mechanisms underlying hybrid necrosis are not well understood. The objective of this study was to conduct a transcriptome analysis of necrotic F_1 wheat plants to better characterize the molecular and genetic mechanisms important to the expression of hybrid necrosis. A total of 3208 genes were differential expressed during the development of hybrid necrotic symptoms. Gene expression changes were detected even before the development of the first visible necrotic symptoms in plants. Rapid changes in gene expression were observed with the initiation of necrosis and the first visual signs of necrotic tissue development. Expressed-gene ontology demonstrated the up-regulation of defense signaling and stress-related genes during the development of hybrid necrosis symptoms. Conversely, photosynthesis-related genes were down-regulated during the development of symptoms. The activation of polyamine oxidase (a source for ROS) and the activation of antioxidant enzymes during the development of necrotic symptoms suggests the involvement of ROS-mediate cell death. There was also activation of CRT3, which is required for the EFR-mediated pathogen associated molecular pattern-triggered immunity (PTI) in plants. In this experiment, the activation of genes encoding for plant recognition

receptors (PRRs), the up-regulation of pathogenesis–related proteins, calcium regulation genes, the activation of plant disease defense related mitogen activated protein kinases (MAP kinases), and the up-regulation of genes involved in ethylene biosynthesis all suggest that the mechanisms responsible for hybrid necrosis are either the same, or are very similar to those activated in response to pathogens, or pests. As has been proposed, the interaction of necrosis genes from both parents of a wheat hybrid may elicit a type of autoimmune response that is similar to how the plant might respond to a pathogen, or pest.

Introduction

Common wheat is an allohexaploid (AABBDD, 2n = 6x = 42) that evolved when the tetraploid wheat (*T. turgidum* AABB genome; emmer and durum wheats) combined with the diploid wheat, *Ae. tauschii* (DD genome; Kihara, 1944; McFadden and Sears, 1944). The chromosome complement was spontaneously doubled in nature, which led to the maintenance of the hexaploid species and the eventual evolution and selection of cultivated, bread wheat (Kihara, 1944; McFadden and Sears, 1944). However, certain interspecific and intraspecific wheat hybridizations can produce abnormal phenotypes that exhibit chlorosis and necrosis (Hermsen 1963a, 1963b; Mizuno et al., 2010). Hybrid necrosis as a result of intraspecific hybridization is more frequent than chlorosis, and it is induced when *Ne1* and *Ne2* genes, both deriving from the B-genome are combined in a hybrid (Nishikawa et al., 1974; Chu et al., 2006; Vikas et al., 2013).

Adaptive evolution, as predicted by the Dobzhansky-Muller theory (Dobzhansky and Dobzhansky 1937, Muller 1942; Orr 1996) has been proposed as a reason for why necrosis genes have been maintained in wheat. The model proposes the reduction of hybrid fitness due to the epistatic interaction involving at least two loci, each of which are derived from divergent parents. An example is found in tomato (*Solanum lycopersicum*). When the *Cf-2* resistant gene against *Cladosporium fulvum* was transferred to the domestic tomato, in which the resistant loci are co-evolved, there is no expression of hybrid necrosis. However, necrosis is expressed when the gene loci are derived from divergent parents (Kruger et al., 2002). Thus, according to the DM model, genetic incompatibility and hybrid necrosis is more likely to occur in polyploid species than in diploid species (Tikhenko et al., 2008; Hatano et al., 2012). DM loci and genes responsible for hybrid incompatibility have been identified in several crop species (Bomblies and Weigel, 2007).

Hypersensitive response mechanisms and the compounds similar to what is observed in responses to pathogens and pests are often involved in the expression of hybrid necrosis. For example there is an increase in the production of ROS in both hypersensitive and hybrid necrotic plant responses (Khanna-Chopra et al., 1998; Sharma et al., 2003; Mizuno et al. 2010). In *Arabidopsis*, necrotic hybrids exhibited increased resistance to a broad spectrum of pathogens (Bomblies et al., 2007), and in another *Arabidopsis* study, hybrid necrosis symptoms were dependent on salicylic acid

production (Alcazar et al., 2009). The activation of pathogen resistance genes *PR1* and *PR5* was demonstrated as essential to the development of necrosis in interspecific tomato hybrids (Masuda et al., 2007). Interspecific wheat hybrids exhibited up-regulation of defense related genes while expressing necrosis (Mizuno et al., 2010), and in the case of an intraspecific wheat hybrid expressing necrosis, some resistant genes were up-regulated along with many stress related genes (Zhang et al., 2014).

The sequencing of the transcriptome and RNA-seq are important applications of Next-Generation Sequencing (NGS) (Mardis, 2008; Wang et al., 2009; Strickler et al., 2012), as the analysis of transcripts is comprehensive and unbiased (Wolf et al., 2013; Conesta et al., 2016). As applied to hybrid necrosis, the analysis of genes that are transcribed can help determine what genes are involved in the response, and how similar the response is to the host reactions to pathogens and pests. Many previous transcriptome analyses were based on the use of a DNA microarray. DNA microarray is restricted to transcripts that have probes in the arrays, and also difficult to use in the accurate detection and quantification of transcriptional activity. (Shendure, 2008; Wang et al., 2009). Though pathogen and pest resistance genes have been documented as being activated in hybrid necrotic responses, a more detailed picture of the genes and transcripts involved is necessary in order to develop effective strategies to mitigate, and possibly avoid hybrid necrosis symptoms in wheat.

Materials and Methods

Plant Material

Hybrid plants derived from a cross between the synthetic hexaploid wheat line 'TA4152-37' and the hard red spring wheat cultivar 'Alsen' were used in this experiment. The harvested F_1 seeds were sown to plastic pots measuring 10 cm x 10 cm and filled with Sunshine Mix 1 (Sun Gro Horticulture Canada Ltd., Seba Beach, AB, Canada). The experiment was conducted by growing plants in an Adaptis Conviron growth chamber (Control Environments Ltd., Winnipeg, Manitoba, Canada) that was maintained at 25 ± 2^{O}C : 20 ± 2^{O}C with a 16 h supplemental light period.

Sampling

All samples were taken from the basal half of the second leaf. The time of sampling was chosen based on the stage of necrotic tissue development in the leaf. Three leaf tissue samples were taken, each at three different times. The first sampels were taken from the second leaves at the first sign of necrotic tissue on the first leaves, and when there was no sign of necrotic tissue on the second leaves (D1). The second samples were taken when the first necrotic tissue was observed at the tips of the second leaves (D2). The third set of samples (D3) was taken when the necrosis covered approximately 50% of the total area of the second leaf (D3). The second leaves were 7, 11, and 16 d old at the D1, D2, and D3 sampling times, respectively. Samples were placed in 15 ml

conical centrifuge tubes (Thermo Fisher Scientific Inc. Waltham, MA, USA) and frozen immediately in liquid N, after which they were stored at -80^{o}C until extraction.

RNA Extraction

RNA was extracted using the TRIzol® reagent according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). The purity and concentration of RNA was determined using a Nanodrop 1000 spectrophotometer (Thermofisher Scientific, Waltham, MA, USA), while the integrity of RNA was confirmed by running extractions on 1% agarose gels, after which samples were sent to the University of Illinois Sequencing Facility for construction of the library, and sequencing.

RNA-Seq Library Construction and Illumina Sequencing

Library construction and sequencing using an Illumina HiSeq2000 were performed at the University of Illinois–Urbana Champaign Sequencing Facilities. A TruSeq^{TM} RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) was used for RNAseq library construction. Sequencing created fastq files which were demultiplexed with the bcl2fastq v1.8.4 conversion software (Illumina, San Diego, CA, USA).

Identification of Differentially-Expressed Genes

Trimming and mapping of reads and statistical analyses were performed using CLC Genomic Workbench 7.5 (Aarhus, Denmark). To obtain high quality reads, raw reads were trimmed using strict filtering criteria. A quality limit value of 0.003 and a read length of >30 nucleotides were used to trim the adapter sequences. Low-quality sequences, empty reads, and tags that were too short were removed through trimming.

The remaining high-quality single end RNA-seq reads from each library were aligned to the wheat reference sequences released by the International Wheat Genome Sequencing Consortium (IWGSC; accessible at http://plants.ensembl.org/Triticum_aestivum). Reads were mapped to the reference sequences using the following parameters; 1) a maximum number of mismatches of 2; 2) a minimum length of fraction of 0.8; 3) a minimum similarity fraction of 0.9; and 4) an unspecific match limit of 10. The expression value of each transcript was normalized to reads per kb per million reads (RPKM). Principal component analysis (PCA) and box plot analyses (BPA) were performed to check the quality of the mapping (Fig. 13 and Fig. 14). Two samples, D2-R1, and D3-R1, to which PCA and BPA were applied were identified as outliers and excluded (Fig. 13, and Fig. 14). Samples D2-R1 and D3-R1 also possessed reads that derived a greater proportion of their sequence from the introns (Table 11). Genes that did not possess the 50 reads in a sample were removed before temporal expression pattern and differential gene expression analyses commenced. Genes

with a false discovery rate (FDR) p–value of less than 0.01, and a fold change of ≥ 2 or ≤ -2, for at least one time point were used for the temporal expression pattern analyses. Differentially-expressed genes were annotated in CLC genomics. MAPMAN (Thimm, Blasing et al. 2004) was used to visualize the functional analysis of differentially-expressed genes.

Results

A total of more than 273 million original sequencing reads were produced from Ilumina sequencing, representing 87,501,820 and 89,470,873, and 96,952,992 raw reads from the library developed from samples D1, D2 and D3, respectively (Table 11). After trimming the low quality reads, 62.5% (D1), 71.89% (D2), and 70.78% (D3) of the cleaned reads were mapped to the reference genome accessible at http://plants.ensembl.org/Triticum_aestivum (Table 11).

Temporally Expressed Gene Patterns

A total of 3208 genes exhibited differential expression patterns to at least one time point (Fold change ≥ 2 and FDR adjusted p <0.01). These 3208 genes were grouped into eight clusters according to their expression patterns (Fig. 15, Clusters 1-8).

Clusters 1, 2, 3, 5, and 7 in Fig. 15 represent the genes mostly down-regulated throughout the sampling period D1 to D3. Among these, clusters 7, 1, and 5 comprise the genes that were highly down-regulated. Genes encoding for the ribulose biphosphate carboxylase (RuBisCo enzyme, rbs1) enzyme, and the genes encoding for the chlorophyll a–b binding proteins were sharply down-regulated in cluster 7. Cluster 2 includes the genes encoding for the RuBisCo enzyme rbs2, along with the chlorophyll a-b binding proteins, and the genes involved in the light harvesting complexes of both photosystem I (PSI) and photosystem II (PSII; LHCA2 and LHB1B1). Genes encoding for glycine-rich RNA binding proteins, and a gene encoding for thiamine biosynthesis were slightly up-regulated from D2 to D3. Cluster 5 includes the genes for both rbs1 and rbs2, the chlorophyll binding a-b proteins, and the light harvesting complexes in both PSI and PSII (LHCA1, LHCB6). Cluster 3 includes many genes from different functional groups that were down-regulation, as indicated by their expression patterns. Along with the genes involved in photosynthesis, genes encoding for the galactose mutarotase-like superfamily protein, and for triose phosphate under were down-regulated. Genes encoding for the phosphoglycerate kinase enzyme, genes associated with the glycine-rich RNA binding protein, and genes for ascorbate peroxidase were downregulated. Genes encoding for magnesium chelatase and the glutamyl-tRNA reductase 1 (GluTR) also exhibited a pattern of down-regulation as depicted in cluster 3. Downregulated, glycine-rich RNA binding proteins at D2 were up-regulated at D3.

Cluster 4 in Fig. 15 represents a group of 29 genes that were upregulated at D1 to D2, before being significantly down-regulated at D3. Among them were genes encoding for fructose-biphosphate, aldolase, glyceraldehyde-3 phosphate dehydrogenase B, and the aldolase superfamily of proteins involved in the Calvin cycle and glycolysis. Genes encoding for the Beta-glucanase isoenzyme and the heat shock protein (HSP82) were upregulated more than most at D2, but HSP82 was down-regulated at D3. Cluster 4 also comprises the genes encoding the glycine decarboxylase P-protein (GLDP2) that are involved in glycine cleavage during photorespiration, as well as the genes encoding water channel protein, RWC1.

Highly up-regulated genes are illustrated as occuring in cluster 8. A gene encoding for calreticulin 3 (CRT3) and a gene encoding for polyamine oxidase (PAO) were steadily upregulated at D1 to D2, and highly upregulatedat D2 to D3. A gene encoding for thea protein associated with the PSII reaction was upregulated as D1, and it was continuous up-regulated at D1 to D3.

Cluster 6 in Fig. 15 includes the largest proportion of genes differentially expressed during the hybrid necrotic response. Thus, cluster 6 was sub-divided into eight sub-clusters (Fig. 16). Generally with few exceptions, upregulated genes throughout the progression of the necrosis are represented in sub-clusters 1, 2, and 3 (Fig. 16).

Sub-clusters 1 and 3 represent highly up-regulated genes at D1 to D3. Sub-cluster 1 includes genes involved in signaling, responses to stresses, ethylene biosynthesis, the

production of secondary metabolites, lipid metabolism, and metabolic transport. The same subcluster also includes the genes encoding for a glycerophosphoryl diester phosphodiesterase like protein, genes encoding for the cysteine synthase enzyme, and also a gene encoding for the glyceraldehyde -3-phosphate dehydrogenase (GADPH) enzyme. In sub-cluster 1, one gene encoding for CRT3 is typically involved in producing calcium signaling products; whereas, there were two genes encoding for the inorganic phosphate transporter (Pht1), and one encoding for the LRR protein kinase family that were the most highly up-regulated.

The largest group of genes included in cluster 3 were those involved in signaling, protein synthesis, protein modification, and stress responses. The largest proportion of signaling genes were from the receptor-like kinase (RLK) family, with genes for calcium signaling making up a smaller proportion. Four of the RLK genes encoding for SNC4, each of the genes encoding for calreticulin 1 (CRT1), calnexin 1 (CNX1), calmodulin 5 (CAM5), and three homologs of calnexins were activated at D1 to D3. Six up-regulated genes related to to the biotic stress response in wheat were expressed in sub-cluster 3. They represent genes encoding RLP46, DAD-1 (defender against cell death), and an osmotin like protein OSM34. Sub-cluster 3 also includes genes encoding the homologs of the heat shock protein, HSP 70. From the oxidation reduction (redox) group, three genes encoding superoxide dismutase 1 (CSD1), and one gene encoding APX1 were also up-regulated at D1 to D3. In addition, sub-cluster 3 includes genes encoding for cellulose

accumulation and pectin linking genes, such as, alpha-1, 4-glucan protein synthase (a cell wall protein); genes involved in flavonoid and phenylpropanoid biosynthesis; five different genes encoding ethylene biosynthesis; and a gene encoding for WRKY 18.

Sub-cluster 2 (Fig. 16) represents a large group of genes that were mostly upregulated during the development of necrotic symptoms. These genes include; 32 associated with biotic stress responses, and 20 associated with abiotic stress responses, all of which were up-regulated at D1 and D3. Other genes typically related to biotic stress responses were up-regulated within the samples analyzed. For example, genes encoding for the the following groups were all up-regulated in the samples taken from hybrid necrotic plants; the disease resistance LRR family of proteins such as NB-LRR proteins, the receptor-like proteins RLP46, RLP15, and RLP7, the VIRB2–interacting proteins BTI1 and BTI3, DAD-1 and DAD-2 (defender against cell death 2), a paralog of NPR1, and two genes encoding for the lipase-like gene important for salicylic acid signaling. Genes for the heat shock proteins (HSP89.1, HSP70, and HSP90), the peroxidase superfamily proteins, and the oligosaccharyl transferase enzyme were also up-regulated at D1 to D3.

The RLKs, calcium- and MAP kinase signaling genes were among the major ones activated at D1 and D3 in sub-cluster 2 (Fig. 16). Wall-associated receptor-like kinase genes (WAK3, WAK5), and the genes encoding PR5–like receptor kinases (PR5K) were activated at D1 to D3. Genes for calcium-dependent protein kinase 3 (CPK3), calcium-

dependent protein kinase 16 (CPK16), calmodulin 5 (CAM5), calmodulin–domain protein kinases (CDPK7, CDPK5), CNX1, MAP kinase kinase 1 (MAPKK1), MAP kinase 5, and MAP kinase 6 were all up-regulated.

Genes encoding for ribosomal protein synthesis represented the largest group of genes upregulated in sub-cluster 2 (Fig. 16). After ribosomal protein synthesis, secondary pathway, protein degradation, and protein modification genes represented the next-largest group of genes exhibiting up-regulation. Redox pathway genes also were up-regulated during the development of necrotic symptoms. Genes encoding for APX1 and CSD1, and a gene encoding for polyamine oxidase (PAO) were upregulated at D1 to D3.

Eight genes involved in ethylene biosynthesis, three associated with abscisic acid synthesis, four encoding for brassinosteroids, and two for jasmonic acid *JAZ1* (lipoxygenase) were the up-regulated. Sub-cluster 2 also includes genes for several families of transcription factors. For example, *MYB, NAC,* and *Snf7* were upregulated at D1 to D3. Moreover, sub-cluster 2 (Fig. 16) also includes the genes involved in the biosynthesis of several secondary metabolites. Among those secondary metabolites were; isoprenoids, phenylpropanoids, alkaloids, flavonoids, chalcones, dihydroflavonols, and flavonols.

Sub-cluster 2 included the up-regulation of a large number of genes involved in transport metabolism. Such genes include those encoding for the transport of amino acids

(cationic amino acid transporter, CAT), calcium (endomembrane type CA–ATPase 4, ECA4), metals, sugar, etc.

Sub-clusters 5 and 6 (Fig. 16) represent a group of genes that were up-regulated at D2 before being down-regulated at D3, as was the case with genes in sub-cluster 8. Genes of sub-cluster 6 included the genes for the aldolase superfamily of proteins and the glycine decarboxylase P–protein 1 (GLDP1), which are important in the Calvin cycle, and for glycolysis. Genes encoding for two heat shock proteins (HSP70, HSP82) were up-regulated at D2, but down-regulated later in the necrotic response. In sub-cluster 8, genes up-regulated include; a gene encoding for the cell–wall plasma membrane linker protein homolog (CWLP), a gene for the acyl hydrolase superfamily protein, three genes involved in transport metabolism, and each of the genes encoding for the NADPH oxidoreductase, and the aldolase TIM barrel family protein. Genes for the chitinase enzyme were up-regulated at D2, but had been down-regulated by D3. Chitinase is involved in ethylene/jasmonic acid mediated signaling during the plant resistance response to a pathogen, or pest.

Sub-cluster 5 includes genes encoding for ribosomal protein synthesis, protein degradation, lipid metabolism, calcium and receptor kinase signaling compounds, and the genes for the synthesis of several secondary metabolites. Sub-cluster 5 also includes the genes encoding for; HSP70, alpha/beta–hydrolases, and the genes involved in fatty acid

biosynthesis, as well as transcription factors, such as; MYB, bHLH, CCCH, and C_2H_2 type zinc finger family proteins.

Sub-cluster 4 in Fig. 16 includes the largest proportion of genes that are involved in important protein pathways. These include the synthesis, degradation, and modification of proteins, as well as genes involved in the metabolism of carbohydrates, lipids, and amino acids. The same cluster includes genes essential for RNA-regulated transcription, signaling, tetrapyrrole synthesis (magnesium chelatase and glutamyl tRNA reductase), cellulose synthesis, and genes involved in plant growth and development. Among the RNA-regulated transcription groups, genes were down-regulated which encode for bHLH, zinc finger (CCCH –type), and MYB transcription factors. Genes ultimately responsible for chlorophyll biosynthesis were universally down-regulated, as represented in sub-cluster 4.

In sub-cluster 7, (Fig. 16), one gene encoding for senescence-associated thiol protease, one gene encoding for aleurain-like protease (ALP), and two genes encoding for $NADP^+$ isocitrate dehydrogenase were highly downregulated at D2. Conversely, these same genes were up-regulated at D3. Two of the genes involved in amino acid metabolism were up-regulated by D3, despite being down-regulation at D2.

Differentially-Expressed Genes in Pairwise Time Comparisons

A total of 919 genes were differentially-expressed before initiation of necrosis (D1) and after initiation of necrosis (D2) in hybrid leaves (Fig. 17). Among them, 395 were up-regulated and 524 were down-regulated. A total of 1692 genes were differentially expressed; 607 were upregulated and 1085 genes were downregulated at D2 to D3 (Fig. 17). The maximum number of differentially-expressed genes was observed when comparing the time points before necrosis began to when the 50% of the leaf exhibited the necrotic symptoms (D1 vs D3).

Change in Gene Expression for Metabolic Functions

Necrosis development in hybrid plants triggered changes in the expression of genes impacting a variety of different metabolic functions (Fig. 18). Photosynthesis-related genes were down-regulated at all time points (Fig. 18). Carbohydrate production and RNA regulation genes were also down-regulated during necrosis development. Tetrapyrrole synthesis-related genes were universally down-regulated, whilst, genes related to stress response pathways were consistently up-regulated (Fig. 18).

Most of the genes that control the expression of enzymes involved in the Calvin cycle were down-regulated as the expression of necrosis progressed (Fig. 19. A. B.C). Furthermore, genes encoding for PSI, PSII, and the RuBisCo enzyme, were

downregulated at D1 to D3 or at D2 to D3 (Fig. 19). Even before necrotic tissue was observed in leaves, genes involved in photosynthesis were down-regulated

Change in Gene Expression at the Cellular Level

At the cellular level, the two largest groups of up-regulated genes were related to biotic and heat stress responses (Fig. 20). The number of genes up-regulated within the biotic stress response group was consistently higher than the number of genes down-regulated for all sample point pairwise comparisons (Fig. 20). As the leaf necrosis developed and and growth slowed, more development-related genes were down-regulated (Fig. 20) Genes encoding for an antioxidant enzyme, such as SOD was initially down-regulated until the onset of necrosis in the second leaf, after which point, genes for SOD were up-regulated as the expression of necrosis progressed (Fig. 20).

Changes in Gene Expression Involving Signaling Mechanisms

Receptor kinase regulated genes were the largest group of genes to be up-regulated, followed by calcium regulating genes (Fig. 21). Genes for MAP kinases were down-regulated until the first expression of necrotic symptoms, but they were up-regulated by D3, when more plant tissue had become necrotic. Genes encoding for ethylene biosynthesis were up-regulated at D2 to D3 and also at D1 to D3 (Fig. 21). Genes involved in the production of brassinosteroids (BA) were upregulated at D1 to D2,

as well as at D1 to D3. Genes encoding for ABA biosynthesis were up regulated at D2 to D3 (Fig. 21).

Change in Expression in Transcription Factors

Genes responsible for the production of transcription factors; WRKY, NAC, and SNF7 were upregulated at D2 to D3, as well as at D1 to D3 (Fig. 22). MYB genes were up-regulated at D1 to D2, and at D1 to D3; however, more MYB genes were down-regulated compared with up-regulated at D2 to D3. Histone family genes were upregulated at D1 to D2; however, they were downregulate at D3. The bZIP family of genes were upregulated at D1 to D2 and D1 to D3, but they were downregulated at D2 to D3 (Fig. 22).

Change in Gene Expression under Receptor Like kinases

The LRR family genes were up-regulated at D1 to D3 (Fig. 23). The LRK10-like and thaumatin genes were also up-regulated, but not as much as LRR genes. Universally up-regulated receptor kinase genes consisted of the S–locus glycoprotein, DUF26, WAK, legume-lectin, and lysine-motif families (Fig. 23).

Changes in Gene Expression Encoding for Secondary Compounds

At D1 to D2, genes encoding for the terpenoids, tocopherol, carotenoids, and cyanogenic glycosides were up-regulated (Fig. 24). However, as necrosis and the expression of necrotic tissue progressed, genes expression for these compounds decreased. Genes encoding phenlypropanoids, lignin, and lignans were up-regulated at D1 to D3; however, more of these genes were down-regulated at D2 to D3 (Fig. 24). Genes encoding for flavonols and dihydroflavonols were upregulated at D2 to D3 (Fig. 24).

Changes in the Expression of Genes Involved in Stress Responses

There was consistent up-regulation of many pathogenesis-related (PR) protein genes at D1 and D3. Most of the genes encoding for the production of signaling compounds (calcium, RCL Kinases, MAP kinases), proteolysis, and heat shock proteins were upregulated at D1 to D3. Genes involved in ethylene biosynthesis were up-regulated as were genes associated with cell wall metabolism, secondary metabolites, oxidation reduction, and abiotic stress responses. Genes encoding for the ABA production were downregulated at D1 to D2. Genes encoding for the jasmonic acid were down-regulated at D1 to D2 (Fig. 25A), but during the interval of D1 to D3 (Fig. 25C), two of the genes encoding for jasmonic acid biosynthesis were up-regulated. Heat shock proteins, including HSP70 and HSP90 were generally up-regulated at D1 to D2 as well as at D1 to

D3 (Fig. 25C). The genes for transcription factor family WRKY were activated at D1 to D3 and at D2 to D3.

The expression analysis of genes for PR proteins is presented in Fig. 26. At D2 to D3, five genes for PR proteins were up-regulated, and up to seven PR proteins were up-regulated at D1 to D3. Genes involved in the production of a variety of different PR proteins were up-regulated at D1 to D3, and all such proteins are typically produced in response to a pathogen, or pest (Fig. 26).

Discussion

Photosynthesis, carbohydrate metabolism, and amino acid metabolic activities are essential to the normal physiological development of plants. Any down-regulation of genes related to these metabolic activities is expected to result in a decrease in the synthesis of energy-storing compounds, as well as the consumption of carbohydrates and amino acids. During photosynthesis, assimilation of CO_2 is catalyzed by the RuBisCo enzyme (ribulose–1, 5–biphosphate carboxylase/oxygenase) (Ellis, 1979). RuBisCo activity in the Calvin cycle has a direct impact on the rate of photosynthesis (Parry et al., 2008). Chlorophyll a-b binding proteins collect and transfer light energy to photosynthetic reaction centers (Xia et al., 2012). Thus, a reduction in the production of RuBisCo enzymes and chlorophyll a-b binding proteins may be the impact of a down-regulation of genes responsible for photosynthesis during wheat hybrid necrosis. Some of

the genes encoding for production of fructose biphosphate, galactose mutarotase, aldolase, glyceral-3-phosphate dehydrogenase, pyruvate dehydrogenase, which are involved in carbohydrate metabolism, glycolysis, and the Calvin and TCA cycles, were up-reguated until the initiation of necrotic symptoms in the second leaf of necrotic hybrids. This suggests that these enzymes, and the genes responsible for their production are functioning to a sufficient level until necrotic symptoms begin to appear. The up-regulation of protein degradation and protein modification-related genes at the onset of necrosis suggests many typical cellular funtions are compromised during the hybrid necrotic response in wheat (Kornitzer and Ciechanover, 2000; Zhang et al., 2014). The down-regulation of genes producing enzymes such as; Glu-tRNA amidotransferase, magnessium chelatase, ferrochelatase, coprorphyrinogen III oxidase, protoporphyrinogen oxidase is an indication that chlorophyll production and the depletion of chlorophyll accompanies the development of necrosis and the development of necrotic tissue in wheat hybrids with complementary *Ne* genes (Tanaka and Tanaka, 2007).

The up-regulation of RLKs and pathogenesis response proteins in hybrid necrotic wheat plants suggests that these compounds are not unique to the pathogen, or pest response, but also are important in the onset and development of hybrid necrosis. Among the RCLs group of genes, more LRR genes were universally up-regulated during the expression of wheat hybrid necrosis. Since plant RLKs are important to the plant response to biotic and abiotic stresses (Padmanabhan et al., 2009; Meng and Zhang,

2013), one can surmise that in wheat hybrid necrosis, the plant recognizes and responds to a complementary *Ne* gene product expressed in the hybrid as if it is responding to a similar gene product produced by a pathogen, or pest. In fact, RLKs and RLPs are associated with pattern recognition receptors (PRRs), which play a key role in the detection of pattern associated molecular patterns (PAMP) or microbe associated molecular patterns (MAMP) during pathogenesis (Meng and Zhang, 2013). Upon recognition of PAMP or MAMP by PRRs, plants can exhibit resistance to a pathogen, or pest by activating its PAMP-triggered immunity (PTI) response (Padmanabhan et al. 2009; Greeff et al. 2012). Two well-known PRRs from the LRR-RLKs groups are EFR and XA21 from *Arabidopsis* and rice, respectively (Holton et al., 2015). EFR-mediated PTI was halted in a *crt3* mutant of *Arabidopsis* (Li et al. 2009a). However, the plants with a normal CRT3 developed EFR-mediated PTI responses (Li et al., 2009a). This demonstrates the important role of CRT3 and PRRs in responding to stress. A gene encoding CRT3 was the most highly upregulated gene during the development of hybrid necrosis in the present study.

Genes for two negative regulators of plant defense (i.e., SNC4, and BAK1 interacting receptor-like kinase BIR; Gao et al., 2009; Bi et al., 2010) were also up-regulated in the present study. The mutant of SNC4, snc4-1D contains a gain of function mutation and encodes defense genes; *PR1*, *PR2*, and *PDF1.2* (Bi et al., 2010). BAK1 is associated with another well known plant recognition receptor FLS2 (Gao et al., 2009).

BAK1 negatively regulates plant R protein-mediated resistance through its interaction of BIR1. In *Arabidopsis,* activation of the defense response and cell death occurred when BIR1function was knocked out in the absence of a pathogen (Gao et al., 2009). Absence of a functional BIR1 activated the *PAD4* and *SOBIR1* resistance pathways, and the activation of defense responses and subsequent cell death took place when the gene for *SOBIR1* was upregulated (Gao et al., 2009).

Along with the up-regulation of genes for RCLs and RLPs involved in PAMP- or MAMP-triggered immunity, the present study also demonstrated the up-regulation of genes for NB-LRR proteins during the expression of hybrid necrosis. NB-LRR proteins are involved in the recognition of pathogen effectors (Nishimura and Dangl, 2010; Meng and Zhang, 2013), and recognition triggers the production of resistant proteins (NB-LRR) and effector triggered immunity (ETI) (Nishimura and Dangl, 2010; Coll et al., 2011, Meng and Zhang, 2013). ETI is part of the defense of plants against specific pathogens, or pests that often results in HR (Nishimura and Dangl, 2010; Coll et al., 2011, Meng and Zhang, 2013).

One of the highly up-regulated genes identified in the present study encodes for the D1 protein associated with plant PSII. Increased irradiation and/or exposure to UV light conditions can damage the D1 protein, resulting in photoinactivation (Garczarek et al., 2008). Degradation of D1 proteins has reportedly protected plants from the damaging effects of light (Garczarek et al., 2008; Huo et al., 2016). In maize (*Zea mays* L.), the

PSBA gene encodes the D1 protein, and this gene is implicated in overcoming drought stresses (Huo et al., 2016). Many heat shock protein genes were upregulated in the present study. Not only are heat shock proteins important to plant responses to heat and drought stress, but they likely are important to responses to cold, wounding, UV light stress, and pathogen stresses (Swindell et al., 2007; Park and Seo, 2015).

It is possible that these same heat shock proteins and the genes producing them are expressed during hybrid necrosis to protect the plant from damaging its own tissue, and specifically protect the plant photosynthetic machinery from significant damage.

Several studies have documented the role of HSP90 and HSP70 in plant immunity responses to microbial pathogens (Kanzaki et al., 2003; Swindell et al., 2007; Park and Seo, 2015). HSP90 triggered R protein activation, and thus, triggered a defense response to microbial pathogens (Liu et al., 2004; Shirasu, 2009; Park and Seo, 2015). The work of Chen et al. (1990) demonstrated the involvement of HSP90 and its co-chaperone Hop/Sti1 in expressing anti-fungal immunity. In *Arabidopsis*, infection by *P. intestans* resulted in the elevated expression of the HSP70 and HSP90 family of proteins (Swindell et al., 2007), and the non-host resistance of *Nicotiana benthamiana* to *Pseudomonas cichorii* was compromised when either the expression of HSP70 or HSP90 was silenced (Kanzaki et al., 2003). The HR reaction was not initiated in *Nicotiana benthamiana* plants, which were silenced for the production of HSP70 or HSP90; whereas, HR was expressed in plants possessing HSP70 and HSP90 (Kanzaki et al., 2003).

Wheat hybrid necrosis and the development of necrotic tissue resulted in the up-regulation of genes for enzymes involved in cellular oxidation reduction reactions. Plants possess several enzymes which produce ROS (i.e., NADPH oxidase, peroxidase, oxalate oxidase, amine oxidases, alternate oxidase, and polyamine oxidases). In the present study, genes for CRT3, the D1 protein, and polyamine oxidase were all upregulated during the expression of hybrid necrosis. The work of Yoda et al. (2006) in tobacco implicated polyamine oxidase in the generation of an oxidative burst and, subsequently, programmed cell death, in reponse to infection by *Phytophthora cryptogea*. Yoda et al. (2003) demonstrated that polyamine degradation by polyamine oxidase produced H_2O_2, which resulted in a hypersensitive reaction in response to the *Tobacco Mosaic Virus*.

The up-regulation of genes producing antioxidant enzymes during the expression of necrosis suggests there was an increased production of ROS during the development of symptoms. Genes for several antioxidant enzymes, such as CSD1, APX1, glutathione-S-transferase (GST) and the genes for the compound glutathione were up-regulated during the development of hybrid necrosis. Superoxide dismutase enzymes convert the superoxide to H_2O_2 (Apel and Hirt, 2004; de Pinto et al., 2015). Sunkar et al. (2006) demonstrated that CSD1 activity increased when plants were exposed to abiotic stresses. The ascorbate peroxidase enzyme, APX1 detoxifies H_2O_2 by converting it to H_2O (Caverzan et al., 2012), and elevated levels of APX1 were reported in rice that was exposed to drought, salinity, and low-temperature stresses (Rosa et al., 2010; Sato et al.,

2011; caverzan et al., 2012). Similarly, detoxification of lipid hydroperoxides were accompanied with the evlevated level of GST during the pathogenesis (Gullner and Komives, 2001; Dean et al., 2005). The glutathione compound also acts as an reactant during the detoxification reaction of H_2O_2 to be catalyzed by the glutathione reductase (Apel and Hirt, 2004).

Along with many changes in the present study, there was up-regulation of genes involved in the calcium homeostasis of the cell. There was the highest up-regulation of gene encoding for the calreticulin3 (CRT3), and also for a gene encoding for calreticulin1 (CRT1). CRT3 defense responses against plant viral and microbial pathogens are documented in several studies (Chen et al., 2005, Li et al., 2009; Saijo et al., 2009), and the CRT1 contribution to the resistance response in biotic and abiotic stress conditions have been noted by Jia et al. (2008). Besides calreticulins, there was the up-regulation of genes encoding for the calcium-dependent protein kinases (CPKs; CPK3, CPK7), the calmodulins (CAMs; CAM5, CAM16), and the Ca^{2+} transporter *CNX1* in the present study. These elevated levels of calreticulins, CPKs, CAMs, and *CNX1* suggest the increased influx of Ca^{2+} into the cell (Cheng et al. 2005; Mahajan and Tuteja, 2007; Jia et al., 2008; Cheval et al., 2013). Increased Ca^{2+} influx into the cytosol is often associated with the plant responses against pathogens and in response to abiotic stresses (Grant et al., 2000; Ranf et al., 2011; Cheval et al., 2013).

In addition to up-regulation of calcium signaling, many genes encoding for MAP kinases signaling were also up-regulated in the present study. There was up-regulation of genes encoding for the MAP kinase6, MAP kinase5, MAP kinase12 and MKK1. Several studies have emphasized the role of MAP kinase3 and MAP kinase6 in plant defenses against many pathogens (Pedley and Martin, 2005; Pitzschke et al., 2009; Tena et al., 2011; Meng and Zhang, 2013). MKK1 acts upstream of MAP kinases and is involved in the signaling of defense against pathogens and abiotic stresses (Kumar et al., 2013; Meng and Zhang, 2013). MAP kinases are important for their functional role in transducing the signals from both PTI and from ETI towards the downstream components that lead to the activation of specific transcription factors, hormones, and the occurrence of defense gene expression in the tissue (Pedley and Martin, 2005; Oh and Martin, 2011; Meng and Zhang, 2013).

Along with the occurrence of many physiological changes, there were also changes in the cell wall and associated metabolisms during hybrid necrosis. Several genes encoding for the WAK2, WAK3, WAK5 and SNC4 were upregulated in the present study. WAKs are linked with cell wall pectin, and play an important role in recognition of pathogen infection. They are also essential for cell wall expansion (Kohorn and Kohorn, 2012; Delteil et al., 2016). During the pathogeneis process, degradation of the cuticle in the cell wall, as well as cell wall products by the pathogen is recognized by the WAKs, which trigger the damage-associated molecular patterns (DAMP) (Ridley et

al., 2001; Brutus et al., 2010). The expression of defense MAP kinases MAP kinase3 and MAP kinase6 were achieved with the the elevation of WAK2 through the pectin treatment in *Arabidopsis* plants (Kohorn and Kohorn, 2012). Similarly, the work of Wagner and Kohorn (2001) indicated the defense role of WAK3 against the pathogens besides its involvement in growth and development. Furthermore, elevated responses of WAK1, WAK2, WAK3 and WAK5 were achieved with the treatment of salicylic acid, suggesting that all these WAKs are involved in defense responses (Wagner and Kohorn, 2001; Sivaguru et al., 2003). Along with WAKs, it is suggested that SNC4 is involved in the perception of pathogen responses. The SNC4 extracellular domain contains the glycerophosphoryl diester phosphodiesterase (GDPD) domain (Bi et al., 2010). This GDPD domain is suggested to perceive the lipid-derived molecules from pathogens, ultimately triggering the PAMP or DAMP (Bi et al., 2010).

In addition to WAKs, gene expression analysis shows the up-regulation of genes encoding for lignin and lignans. This suggests lignification in the cell wall as the plant responds to the onset of necrosis. Lignification, lignin or lignin -like phenolic compound accumulation provides cell wall resistance to mechanical pressure (Vance et al., 1980; Southerton and Deverall, 1990). Lignin also could create a hydrophobic environment in the papilla of the cell wall, which reduces activities of cell wall-degrading enzymes secreted by pathogens (Huckelhoven et al., 1999; Huckelhoven, 2007). Similarly, there was up-regulation of genes encoding for glycerophosphoryl diester phosphodiesterase-

like protein SHV3, and one of its paralogs SVL1. SHV3 and SVL1 are essential for cellulose accumulation in cell walls (Hayashi et al., 2008). SHV3 and related proteins are also required for pectin network formation at the plasma membrane (Jiang et al., 2005; Iwai et al., 2006). Along with lignfication, expression of these genes suggest the cellulose accumulation and pectin linking during hybrid necrosis in the present study. Lignification, deposition of cellulose, and pectin linking all function as a physical barrier against the penetration by pathogens (Vance et al., 1980; Southerton and Deverall, 1990; Bechinger et al., 1999; Jiang et al., 2005; Iwai et al., 2006).

Among the transcription factors, WRKY, MYB, NAC, bZIP and SNF7 were most highly-activated in this study. The majority of WRKY gene products regulate biotic stress responses. Much crosstalk between the MYB, bZIP, and NAC pathways, which are involved in regulation of both biotic and abiotic responses takes place (Ambawat et al., 2013; Rajeb et al., 2014).

Among hormone metabolism, genes encoding for ethylene biosynthesis were the most highly up-regulated. Along with ethylene, genes encoding for the JAZ1 were also up-regulated in the present study. The up-reguation of ethylene/jasmonate pathway in the present study suggests the genetic responses were similar to those of the host to pathogen attacks (Nuruzzaman et al., 2015; Pandey and Somssich, 2009). Up-regulation of genes encoding for ABA suggest that some of the genes and their responses are similar to the plant response to an abiotic stress (Nuruzzaman et al., 2015; Pandey and Somssich,

2009). Up-regulaion of genes encoding for BA also implicates this hormone in hybrid necrosis. Though the mechanism of BA production has not been elucidated, the BA pathway in plants has a role in response to high temperature, along with other abiotic and biotic stress responses (Choudhary et al., 2012; Fariduddin et al., 2014). BA frequently interacts with ABA to influence stress responses in plants (Choudhary et al., 2012).

During growth and development under different environmental conditions, plants produce secondary metabolites that help them to adapt to the environment and also to particular stress conditions (Akula and Ravishankar, 2011). In the present study, genes encoding for the many secondary metabolites i.e., tocopherol, terpenoids, phenylpropanoids, flavonoids, dihydroflavonols, betains were up-regulated. Tocopherol has been implicated in reducing the ROS levels [singlet oxygen ($^1O_2$) and OH^-], and thus is important for minimizing oxidative damage (Munne–Bosch, 2005). Many terpenoids are volatile in nature, and some act as phytoalexins involved in the direct defense against insect herbivores and pathogens (Cheng et al., 2007). Phenylpropanoids, flavonoids, dihydroflavonols are the important compounds that act as antimicrobial and antioxidant agents (Tian et al., 2015). Betains also are involved in a resistance response against pathogens, as well as a response to abiotic conditions such as; frost, heat damage, and saline soil (Blunden, 2003). Thus, in the present study, it appears that cell processes are up-regulated resulting in products that are antioxidant and antimicrobial in nature.

In the present study and in the absence of any significant stress, genes typically functioning in response to biotic and abiotic stresses were upregulated. This highlights the idea proposed by Bomblies et al. (2007), as well as others that plant hybrid necrosis is an autoimmune type of response. As such, some hybrid plants will react as they would to a pathogen or pest when there is recognition of genetic incompatibility, and subsequently, there will be activation of physiological processes that ultimately result in the death of host plant cells. The activation of polyamine oxidase as source of ROS, antioxidant enzyme activities, and cell death suggest the overproduction of ROS impacts the development of a toxic environment, ultimately leading to cell death (Yoda et al., 2003, 2006; Apel and Hirt, 2004). Similarly, the up-regulation of a gene for CRT3, which is required for EFR mediated PTI immunity (Li et at., 2009a), suggests the involvement of defense genes in hybrid necrosis. ROS generation, some Ca^{2+}, and MAP kinases are involved in common mechanisms of plant responses to biotic and abiotic stress (Apel and Hirt, 2004; Mahajan and Tuteja, 2007; Cheval et al., 2013; Meng and Zhang, 2013).

The present study demonstrated the up-regulation of biotic and abiotic stress-related response genes during the expression of hybrid necrosis in wheat. In the case of certain necrotic wheat hybrids, RNA-seq results also support the concept that the expression of this hybrid necrosis is much like an autoimmunity type of response.

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Table 11. Statistics of the illumina, trimmed reads and percent of mapping

Name	Number of reads	Number of reads after trim	Number of mapped reads	Mapped reads (%)
D1-R1	27,834,318	19,437,730	13,490,624	69.4
D1-R2	27,513,997	18,976,855	12,097,121	63.75
D1-R3	32,153,505	21,263,099	11,559,414	54.36
D2-R1	30,676,140	22,212,330	17,422,024	78.43
D2-R2	31,000,887	21,964,829	15,111,392	68.8
D2-R3	27,793,846	19,743,019	13,513,474	68.45
D3-R1	28,040,363	19,868,256	15,218,095	76.60
D3-R2	32,912,965	22,783,840	15,426,741	67.71
D3-R3	35,999,664	25,258,633	17,184,914	68.04
Total	273, 925, 685	191, 508,591	131, 023, 799	68.39

(D1, D2 and D3 are the sampling dates, D1 = samplings before the onset of necrosis in second leaf, D2 = samplings taken with the beginning of necrosis in second leaf, and D3 = samplings taken when the necrosis covered ~ 50% of the second leaf area. R1 – R3 = Replications)

Fig. Legends

Fig. 13. Principal component analysis (PCA) of the transcriptomic dataset. Left PCA picture is with all the transcripts from nine samples, three of each samples from three time points i.e., D1, D2 and D3. Right PCA is after excluding the two outliers each from D2 and D3. D1 = before the onset of necrosis in second leaf, D2 = beginning of necrosis in second leaf, and D3 = when the necrosis covered ~ 50% of the second leaf area

Fig. 14. Box plot analysis of the transcriptomic dataset. Left picture is with all the transcripts from nine samples, three of each samples from three time points i.e., D1, D2 and D3. Right picture is after excluding the two outliers each from D2 and D3. D1 = before the onset of necrosis in second leaf, D2 = beginning of necrosis in second leaf, and D3 = when the necrosis covered ~ 50% of the second leaf area

Fig. 15. Temporal expression patterns of differentially expressed genes in different clusters. A total of 3208 differentially expressed genes were divided into eight clusters. D1 = before the onset of necrosis in second leaf, D2 = beginning of necrosis in second leaf, and D3 = when the necrosis covered ~ 50% of the second leaf area. False discovery rate (FDR) p <0.01 and fold change ≥ 2 $or \leq$ -2 at least one time point were used to identify the differentially expressed genes.

Fig. 16. Temporal expression patterns of differentially expressed genes in different sub-clusters. A total of eight sub-clusters were designed from the 2886 differentially expressed genes comprised in cluster 6. D1 = before the onset of necrosis in second leaf, D2 = beginning of necrosis in second leaf, and D3 = when the necrosis covered ~ 50% of the second leaf area. False discovery rate (FDR) p <0.01 and fold change ≥ 2 $or \leq$ -2 at least one time point were use to identify the differentially expressed genes.

Fig. 17. Number of differential expressed genes in different time point comparisons in hybrid necrosis of wheat. False discovery rate (FDR) $p < 0.01$ and fold change $\geq$ 2 or $\leq$ -2 were used to identify the differentially expressed genes. Up-regulated and down-regulated genes are on D2 over D1, D3 over D2 or D3 over D1. D1 = before the onset of necrosis in second leaf, D2 = beginning of necrosis in second leaf, and D3 = when the necrosis covered ~ 50% of the second leaf area. False discovery rate (FDR) $p < 0.01$ and fold change ≥ 2 or $\leq$ -2 were used to identify the differentially expressed genes.

Fig. 18. Change in gene expression in different metabolic functions compared at different stages of necrosis in hybrid necrosis of wheat. Up-regulated and down-regulated genes are on D2 over D1, D3 over D2 or D3 over D1. D1 = before the onset of necrosis in second leaf, D2 = beginning of necrosis in second leaf, and D3 = when the necrosis covered ~ 50% of the second leaf area. False discovery rate

(FDR) $p < 0.01$ and fold change ≥ 2 or ≤ -2 were used to identify the differentially expressed genes.

Fig. 19. Photosynthetic pathway overview of differentially expressed genes at different stages of necrosis in hybrid necrosis of wheat. A) D1 vs D2, B) D2 vs D3, and C) D1 vs D3. Blue color indicates the up-regulation and red color indicates the down-regulation of genes. D1 = before the onset of necrosis in second leaf, D2 = beginning of necrosis in second leaf, and D3 = when the necrosis covered ~ 50% of the second leaf area. False discovery rate (FDR) $p < 0.01$ and fold change ≥ 2 or ≤ -2 were used to identify the differentially expressed genes.

Fig. 20. Change in gene expression in different cellular responses compared at different stages of necrosis in hybrid necrosis of wheat. Up-regulated and down-regulated genes are on D2 over D1, D3 over D2 or D3 over D1. D1 = before the onset of necrosis in second leaf, D2 = beginning of necrosis in second leaf, and D3 = when the necrosis covered ~ 50% of the second leaf area. False discovery rate (FDR) $p < 0.01$ and fold change ≥ 2 or ≤ -2 were used to identify the differentially expressed genes.

Fig. 21. Change in gene expression under different cell regulation processes compared at different stages of necrosis in hybrid necrosis of wheat. False discovery rate (FDR) $p < 0.01$ and fold change ≥ 2 or ≤ -2 were used to identify the differentially expressed genes. Up-regulated and down-regulated genes are on

D2 over D1, D3 over D2 or D3 over D1. D1 = before the onset of necrosis in second leaf, D2 = beginning of necrosis in second leaf, and D3 = when the necrosis covered ~ 50% of the second leaf area. False discovery rate (FDR) $p <$ 0.01 and fold change ≥ 2 or ≤ -2 were used to identify the differentially expressed genes.

Fig. 22. Change in expression of different transcription factors compared at different stages of necrosis in hybrid necrosis of wheat. Up-regulated and down-regulated genes are on D2 over D1, D3 over D2 or D3 over D1. D1 = before the onset of necrosis in second leaf, D2 = beginning of necrosis in second leaf, and D3 = when the necrosis covered 50% of the second leaf area. False discovery rate (FDR) $p < 0.01$ and fold change ≥ 2 or ≤ -2 were used to identify the differentially expressed genes.

Fig. 23. Change in gene expressions under the groups of receptor like kinases (RCLs) compared at different stages of necrosis in hybrid necrosis of wheat. Up-regulated and down-regulated genes are on D2 over D1, D3 over D2 or D3 over D1. D1 = before the onset of necrosis in second leaf, D2 = beginning of necrosis in second leaf, and D3 = when the necrosis covered ~ 50% of the second leaf area. False discovery rate (FDR) $p < 0.01$ and fold change ≥ 2 or ≤ -2 were used to identify the differentially expressed genes.

Fig. 24. Change in the production of secondary metabolites compared at different stages of necrosis in hybrid necrosis of wheat. Up-regulated and down-regulated genes are on D2 over D1, D3 over D2 or D3 over D1. D1 = before the onset of necrosis in second leaf, D2 = beginning of necrosis in second leaf, and D3 = when the necrosis covered ~ 50% of the second leaf area

Fig. 25. Biotic stress pathway overview of differentially expressed genes at different stages of necrosis in hybrid necrosis of wheat. A) D1 vs D2, B) D2 vs D3, and C) D1 vs D3. Blue color indicates the up-regulated and red color indicates the down-regulated genes on D2 over D1, D3 over D2 or D3 over D1. D1 = before the onset of necrosis in second leaf, D2 = beginning of necrosis in second leaf, and D3 = when the necrosis covered ~ 50% of the second leaf area. False discovery rate (FDR) $p < 0.01$ and fold change ≥ 2 or ≤ -2 were used to identify the differentially expressed genes.

Fig. 26. Heatmap of differentially expressed pathogenesis related proteins (PR–proteins) compared at different stages of necrosis in hybrid necrosis of wheat. Gene annotations are on the right of heatmap. D1 = before the onset of necrosis in second leaf, D2 = beginning of necrosis in second leaf, and D3 = when the necrosis covered ~ 50% of the second leaf area. False discovery rate (FDR) $p < 0.01$ and fold change ≥ 2 or ≤ -2 were used to identify the differentially expressed genes.

Figures

Fig. 13.

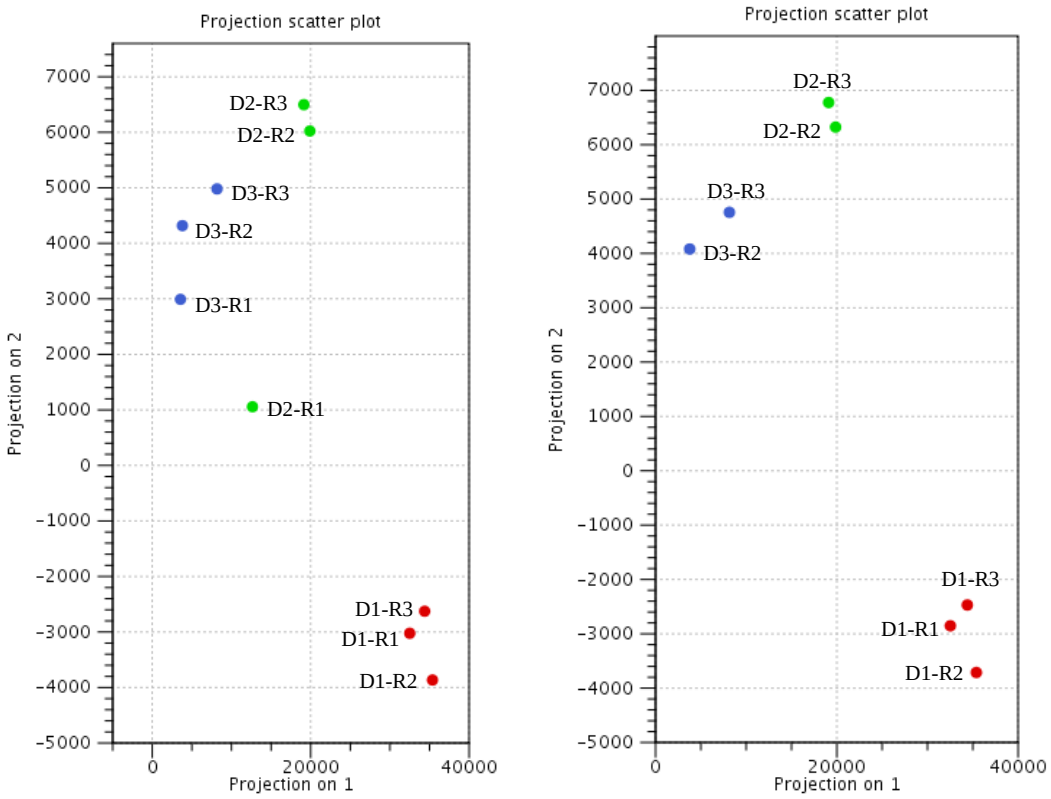


Fig. 14.

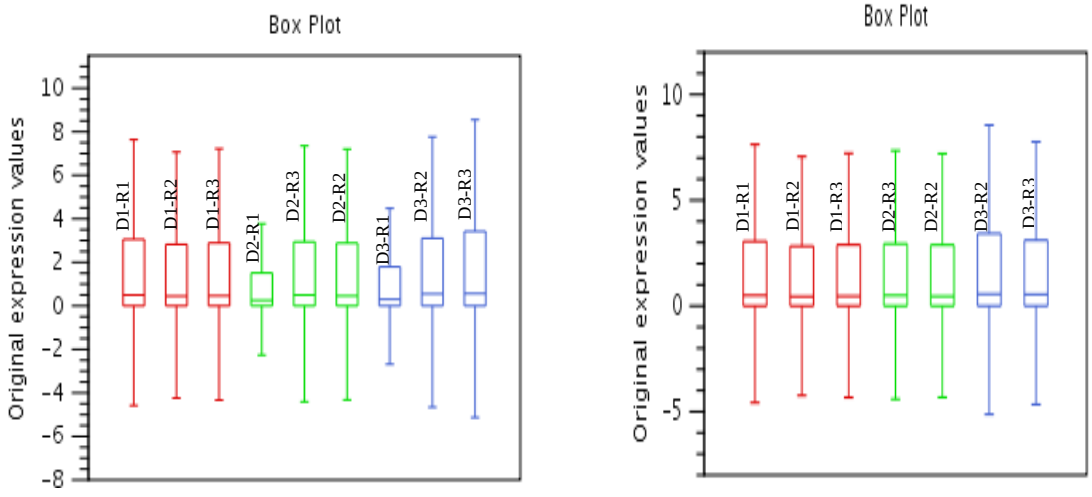

Box Plot
Original expression values
10
8
6
4
2
0
-2
-4
-6
-8
D1-R1
D1-R2
D1-R3
D2-R1
D2-R3
D2-R2
D3-R1
D3-R2
D3-R3
Box Plot
Original expression values
10
5
0
-5
D1-R1
D1-R2
D1-R3
D2-R3
D2-R2
D3-R2
D3-R3

Fig. 15.

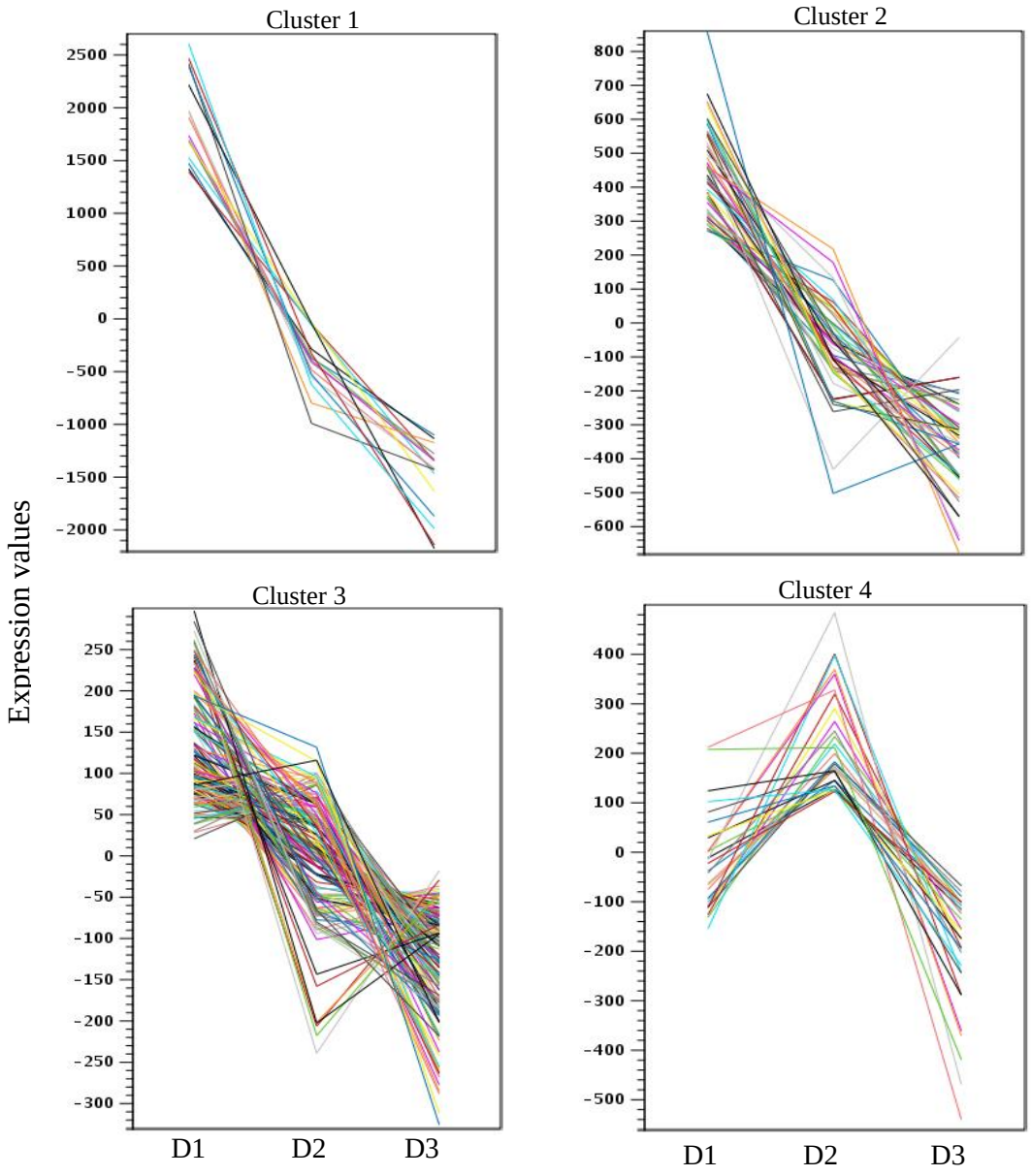

Cluster 1
Cluster 2
Cluster 3
Cluster 4
Expression values
2500
2000
1500
1000
500
0
-500
-1000
-1500
-2000
800
700
600
500
400
300
200
100
0
-100
-200
-300
-400
-500
-600
250
200
150
100
50
0
-50
-100
-150
-200
-250
-300
400
300
200
100
0
-100
-200
-300
-400
-500
D1
D2
D3
D1
D2
D3

Fig.15..

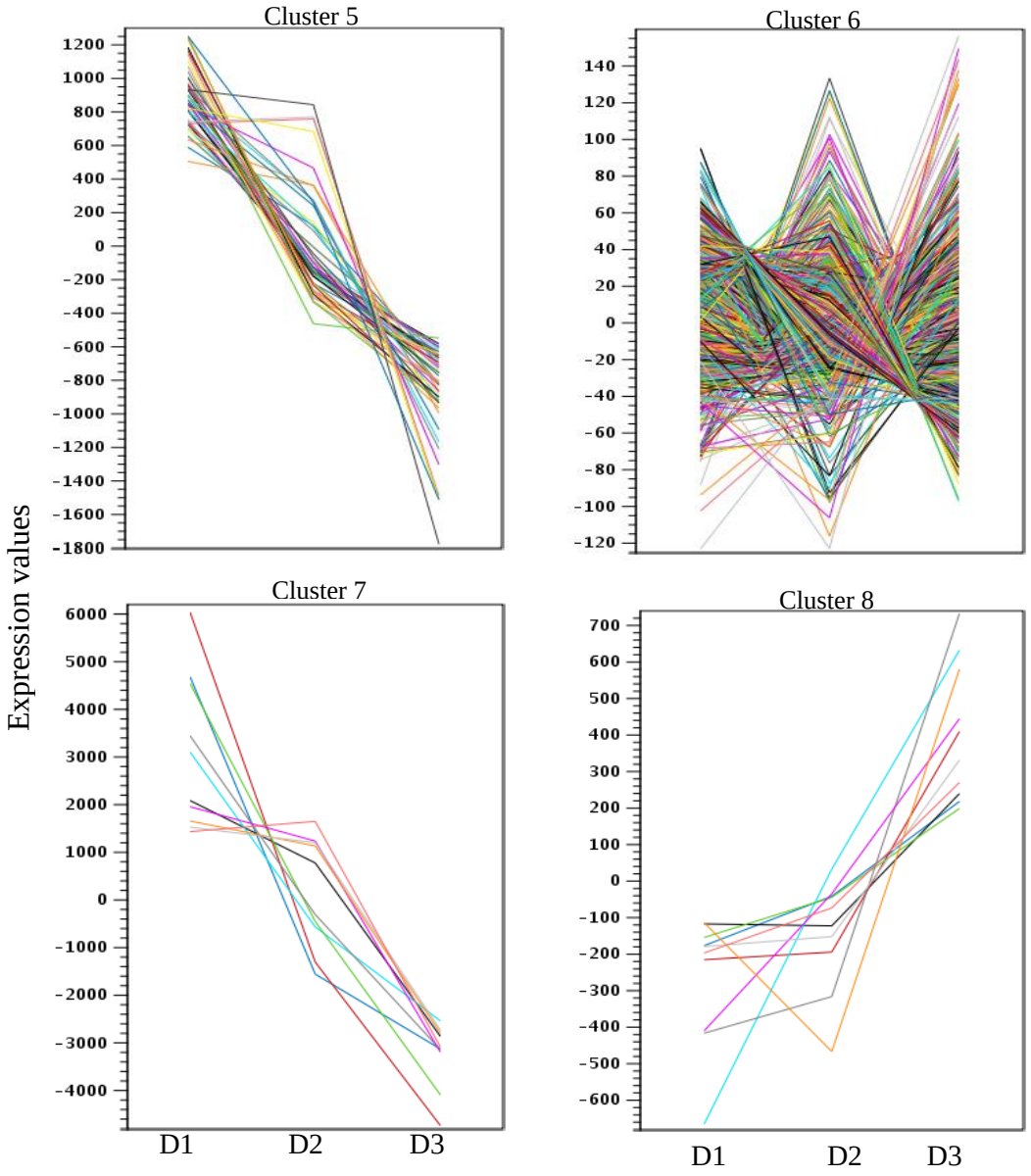

Cluster 5
Cluster 6
Cluster 7
Cluster 8
Expression values
D1
D2
D3
D1
D2
D3

Fig. 16.

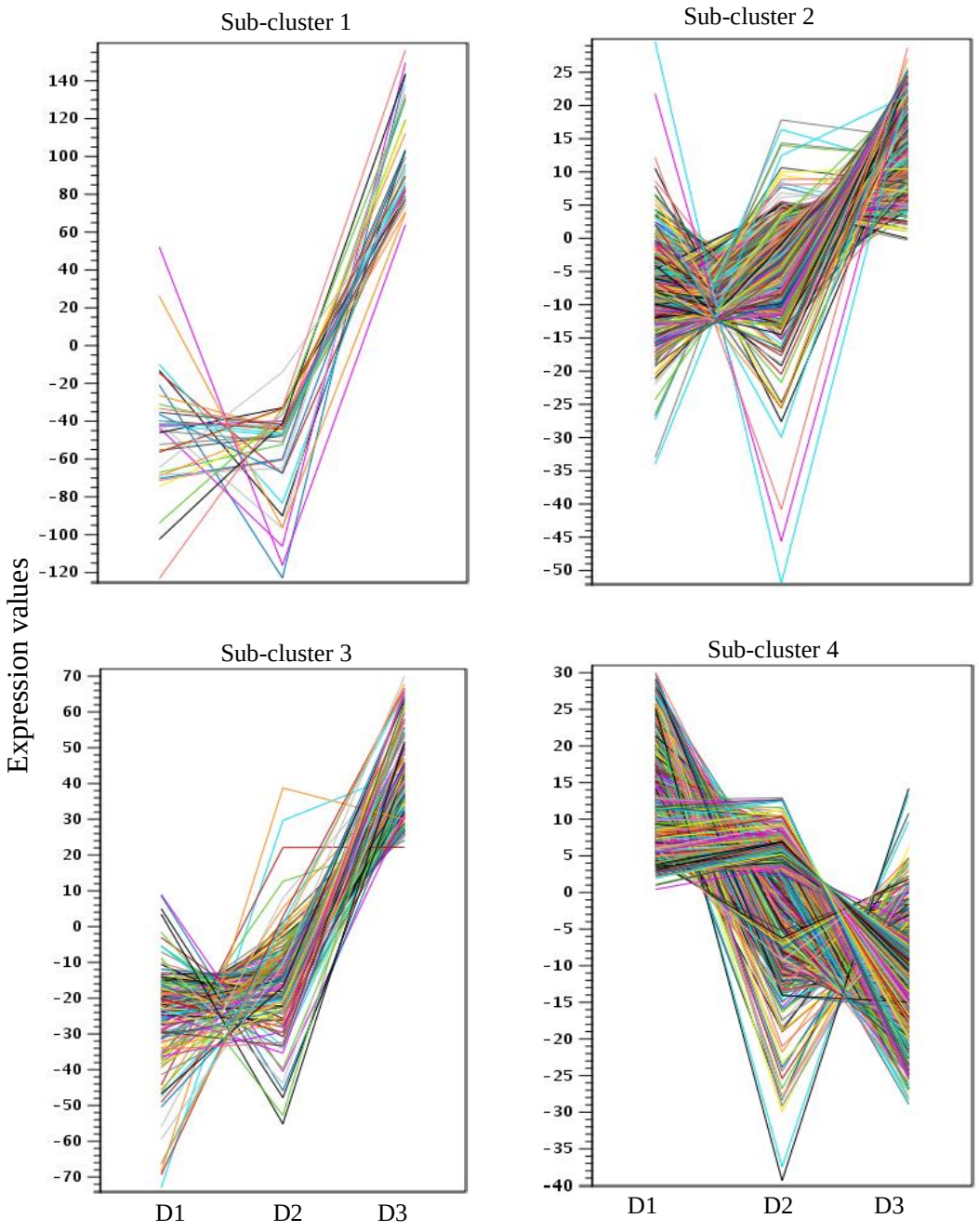

Sub-cluster 1
Sub-cluster 2
Sub-cluster 3
Sub-cluster 4
Expression values
D1
D2
D3

Fig. 16.

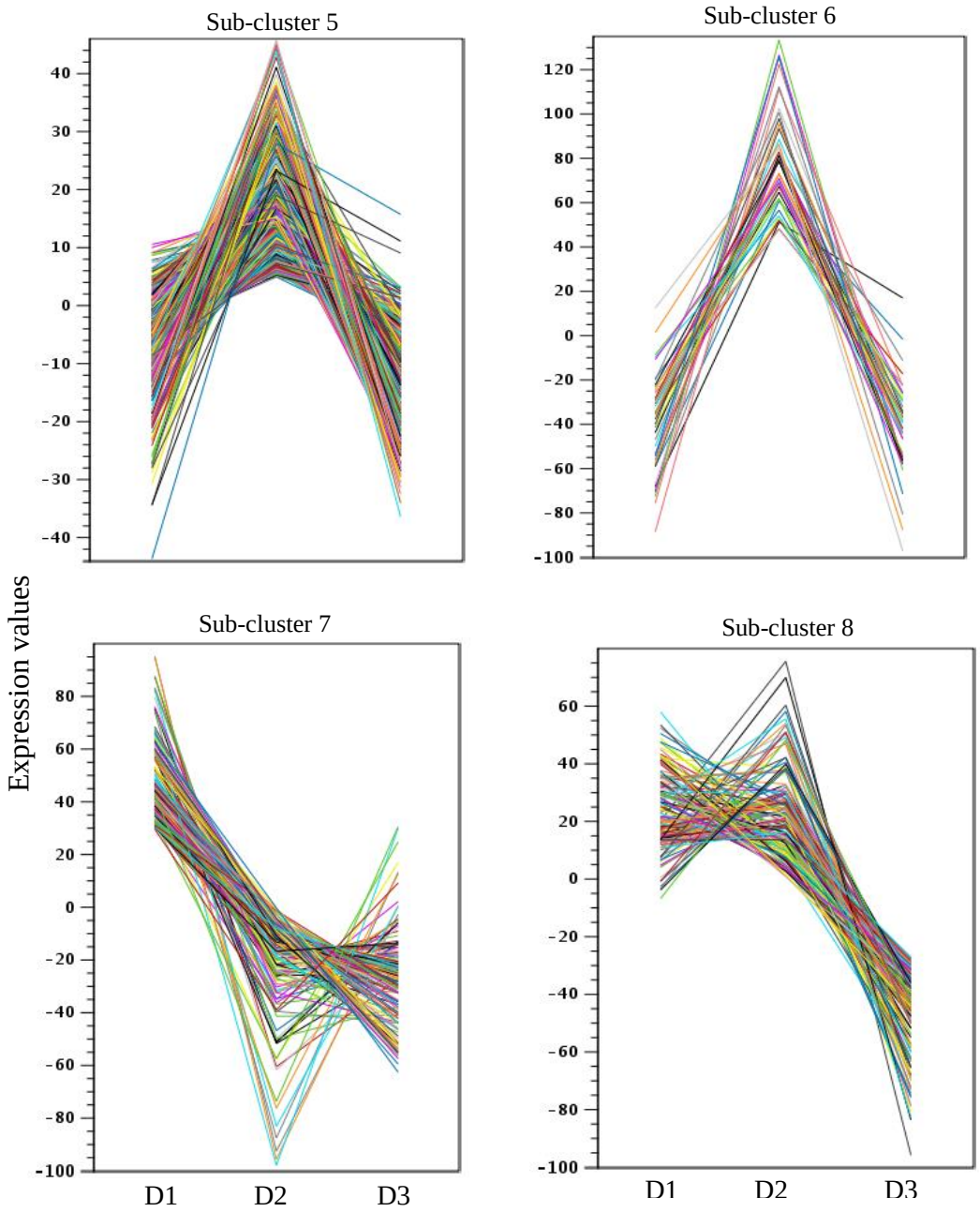

Sub-cluster 5
Sub-cluster 6
Sub-cluster 7
Sub-cluster 8
Expression values
D1
D2
D3

Fig. 17.

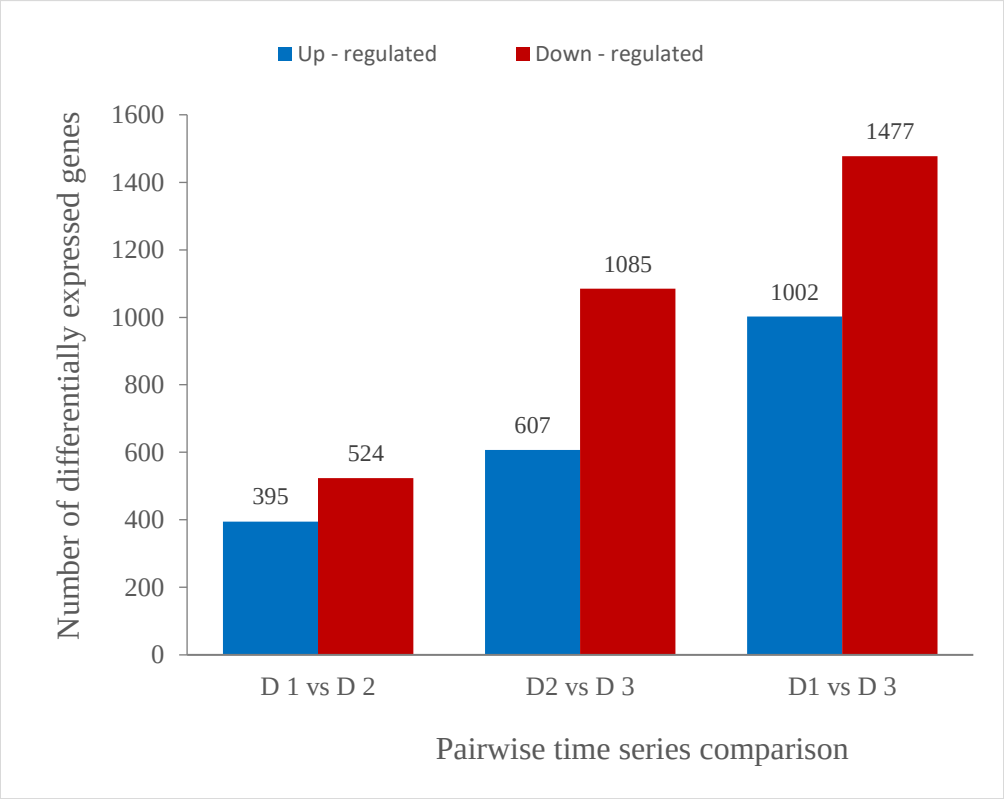
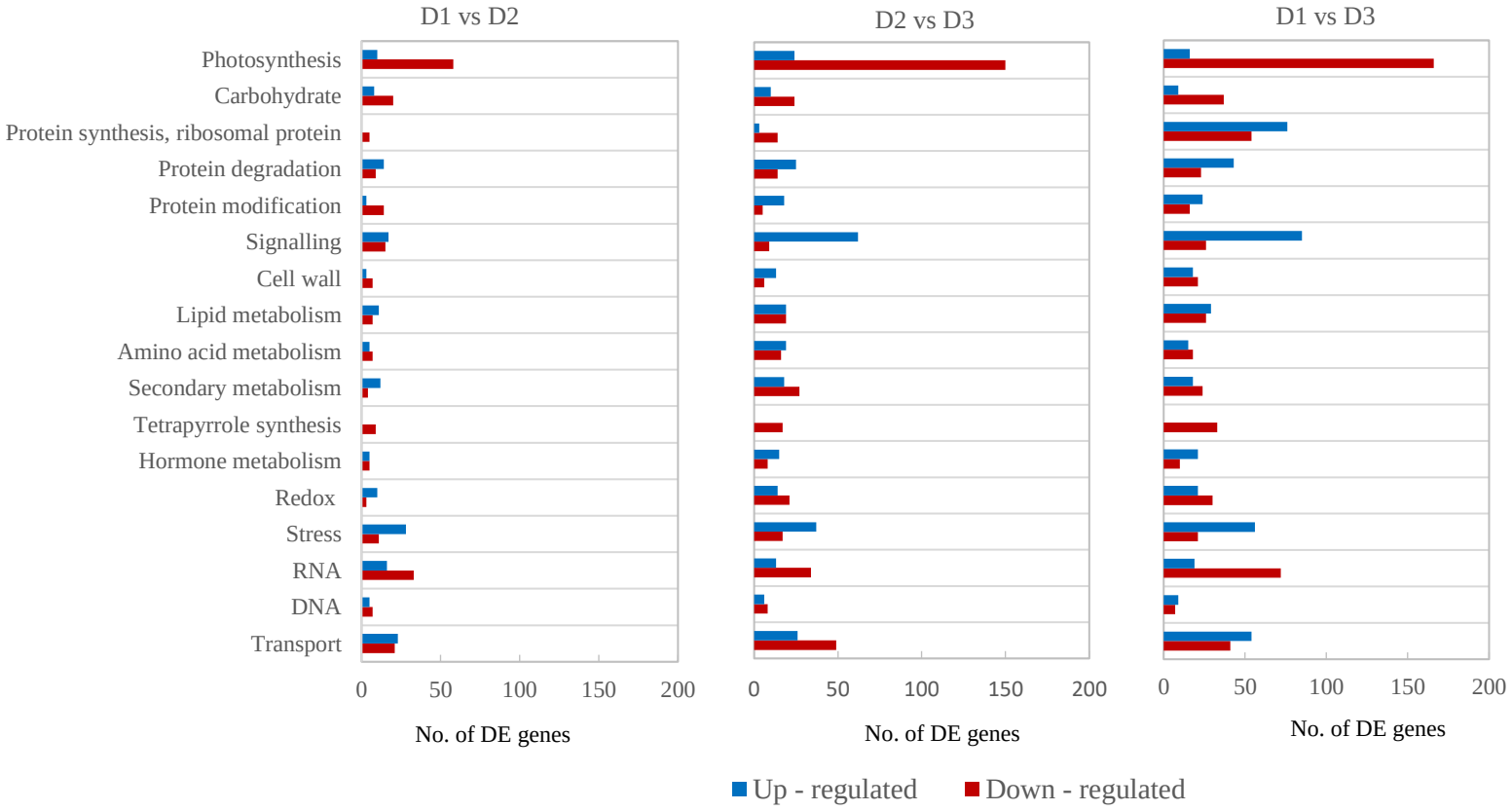

Up - regulated
Down - regulated
Number of differentially expressed genes
1600
1400
1200
1000
800
600
400
200
0
395
524
607
1085
1002
1477
D 1 vs D 2
D2 vs D 3
D1 vs D 3
Pairwise time series comparison

Fig. 18.



(Note: DE = differentially expressed)

Fig. 19.A.

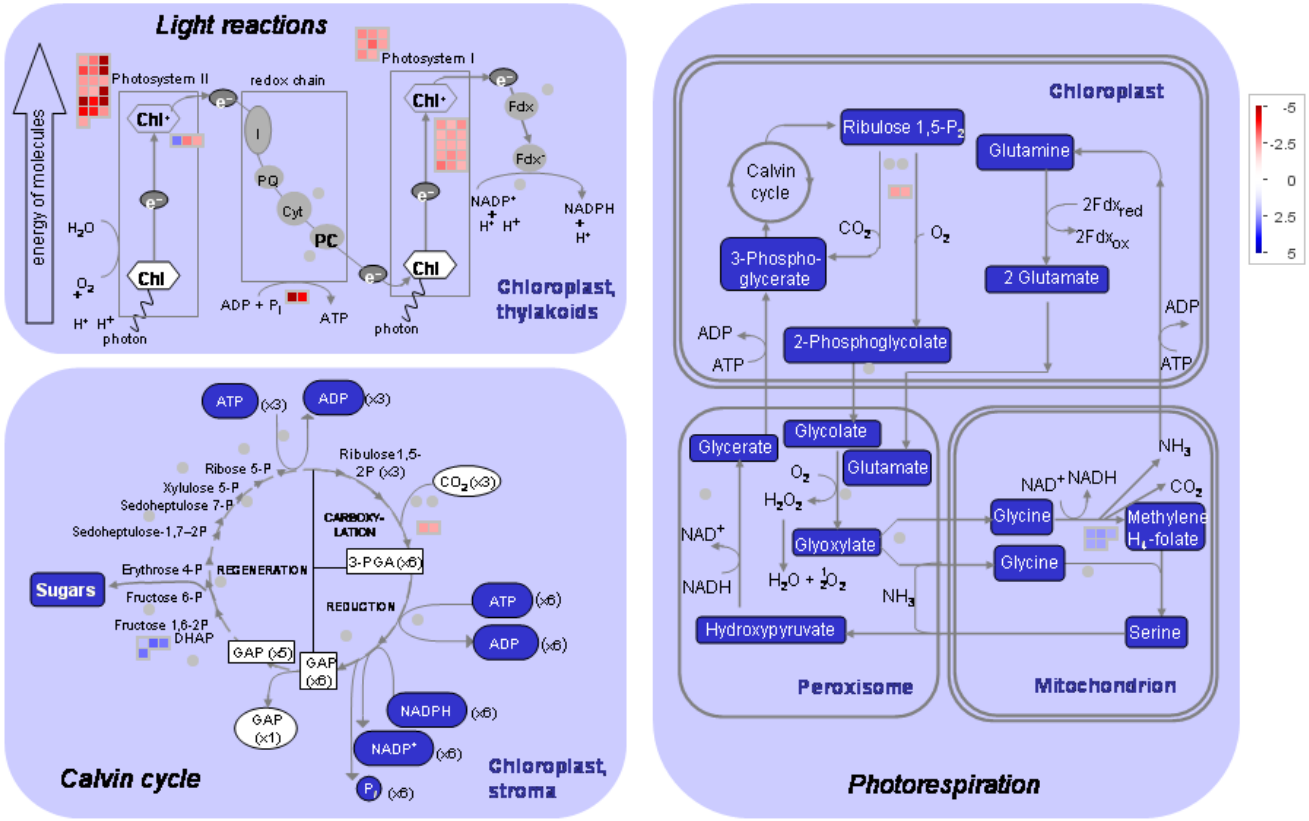

Light reactions
energy of molecules
Photosystem II
redox chain
Photosystem I
Chl*
Chl
e⁻
I
PQ
Cyt
PC
Fdx
Fdx⁻
NADP⁺ + H⁺ H⁺
NADPH + H⁺
H₂O
O₂ + H⁺ H⁺
photon
ADP + Pi
ATP
Chloroplast, thylakoids
ATP (×3)
ADP (×3)
Ribose 5-P
Xylulose 5-P
Sedoheptulose 7-P
Sedoheptulose-1,7–2P
Erythrose 4-P
Fructose 6-P
Fructose 1,6-2P
DHAP
Sugars
REGENERATION
Ribulose1,5-2P (×3)
CO₂ (×3)
CARBOXY-LATION
3-PGA (×6)
REDUCTION
ATP (×6)
ADP (×6)
GAP (×5)
GAP (×6)
GAP (×1)
NADPH (×6)
NADP⁺ (×6)
Pi (×6)
Calvin cycle
Chloroplast, stroma
Chloroplast
Ribulose 1,5-P2
Glutamine
Calvin cycle
CO₂
O₂
2Fdx red
2Fdx ox
3-Phospho-glycerate
2 Glutamate
ADP
ATP
2-Phosphoglycolate
Glycerate
Glycolate
Glutamate
O₂
H₂O₂
NAD⁺
NADH
Glyoxylate
H₂O + ½O₂
NH₃
Hydroxypyruvate
Glycine
Serine
NAD⁺ NADH
CO₂
Methylene H4-folate
Peroxisome
Mitochondrion
Photorespiration
-5
-2.5
0
2.5
5

Fig. 19.B.

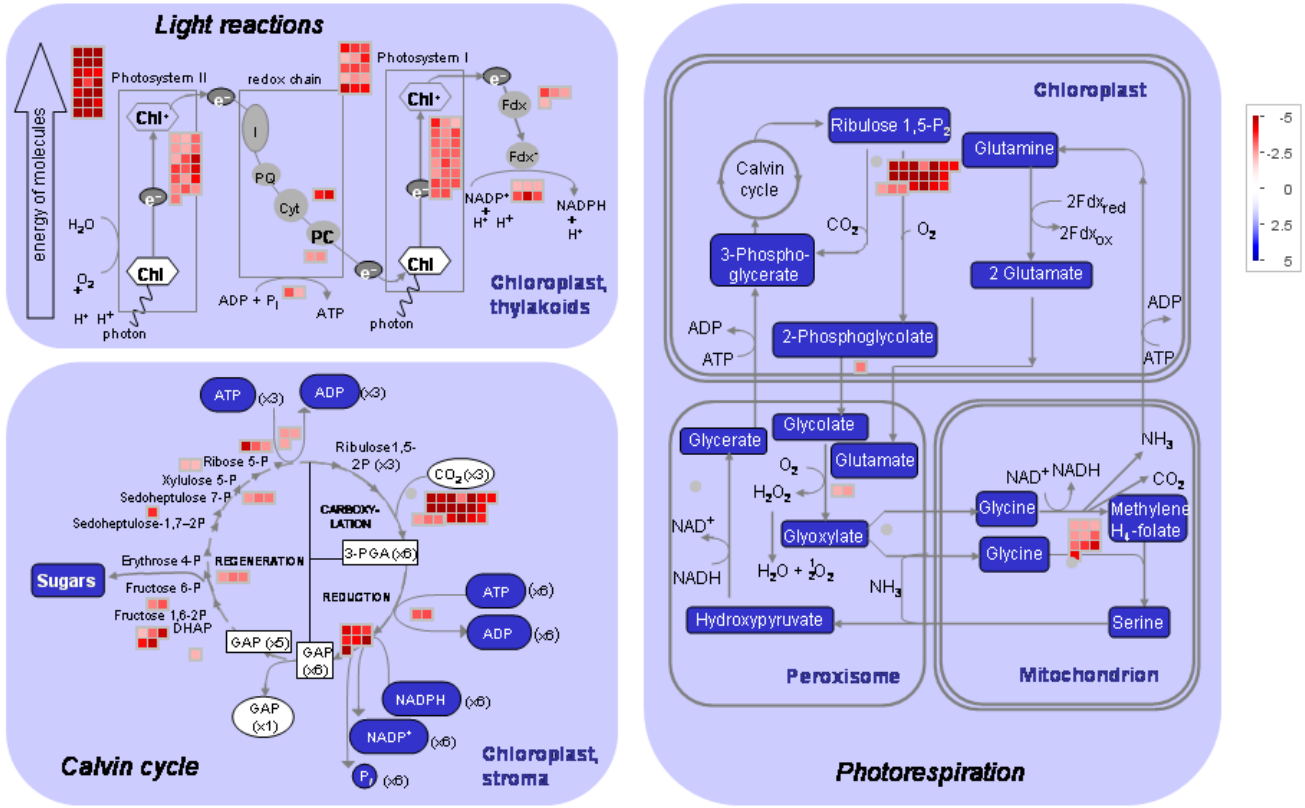

Light reactions
energy of molecules
Photosystem II
redox chain
Photosystem I
Chl*
Chl
e⁻
I
PQ
Cyt
PC
Fdx
Fdx⁻
NADP⁺ + H⁺ H⁺
NADPH + H⁺
H₂O
O₂ + H⁺ H⁺
photon
ADP + Pi
ATP
Chloroplast, thylakoids
ATP (×3)
ADP (×3)
Ribulose1,5-2P (×3)
CO₂ (×3)
Ribose 5-P
Xylulose 5-P
Sedoheptulose 7-P
Sedoheptulose-1,7–2P
CARBOXY-LATION
3-PGA (×6)
Erythrose 4-P
REGENERATION
Sugars
Fructose 6-P
Fructose 1,6-2P
DHAP
REDUCTION
ATP (×6)
ADP (×6)
GAP (×5)
GAP (×6)
GAP (×1)
NADPH (×6)
NADP⁺ (×6)
Pi (×6)
Calvin cycle
Chloroplast, stroma
Chloroplast
Ribulose 1,5-P2
Glutamine
Calvin cycle
CO₂
O₂
2Fdx_red
2Fdx_ox
3-Phospho-glycerate
2 Glutamate
ADP
ATP
2-Phosphoglycolate
Glycerate
Glycolate
Glutamate
O₂
H₂O₂
NAD⁺
NADH
Glyoxylate
H₂O + ½O₂
NH₃
Glycine
NAD⁺ NADH
CO₂
Methylene H4-folate
Hydroxypyruvate
Serine
Peroxisome
Mitochondrion
Photorespiration
-5
-2.5
0
2.5
5

Fig. 19.C.

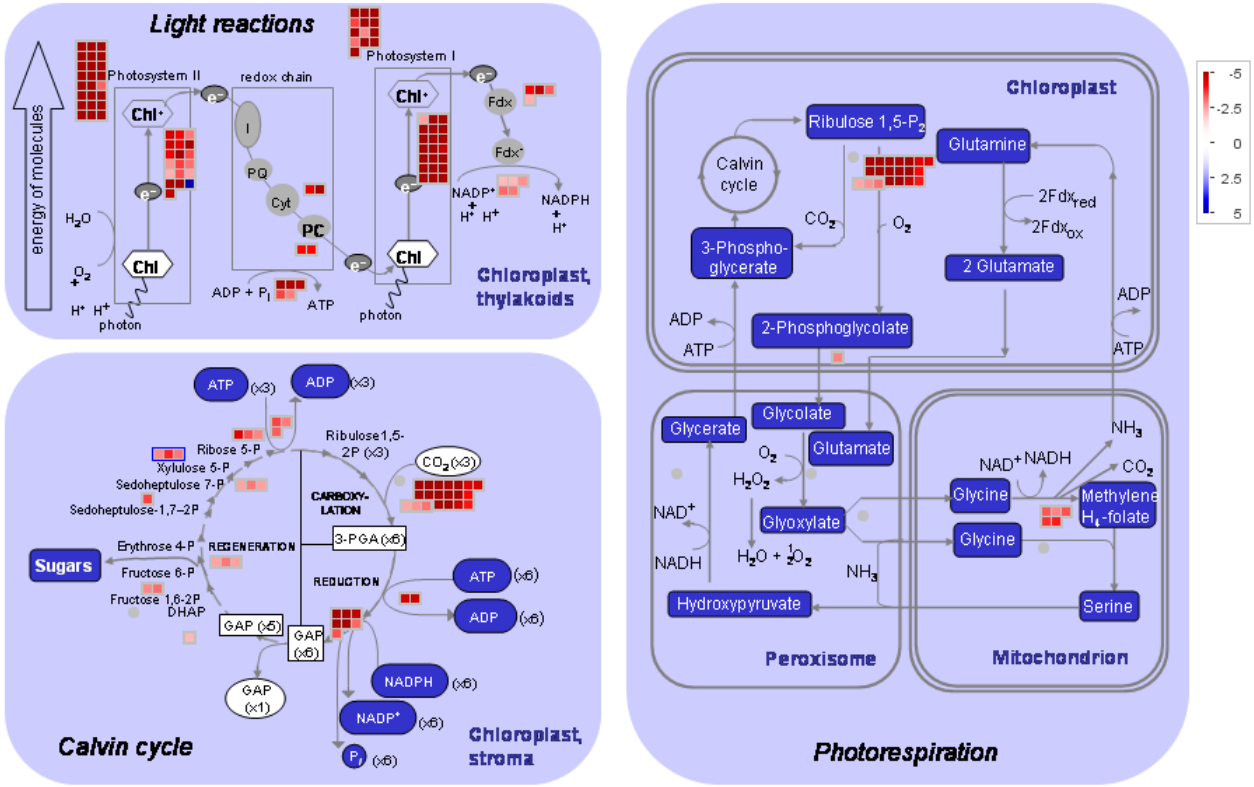

Light reactions
energy of molecules
Photosystem II
redox chain
Photosystem I
Chl*
Chl
PQ
Cyt
PC
Fdx
Fdx-
NADP+ + H+
NADPH + H+
H2O
O2 + H+
H+
photon
ADP + Pi
ATP
Chloroplast, thylakoids
ATP (x3)
ADP (x3)
Ribulose1,5-2P (x3)
CO2 (x3)
Ribose 5-P
Xylulose 5-P
Sedoheptulose 7-P
Sedoheptulose-1,7-2P
CARBOXY-LATION
3-PGA (x6)
Erythrose 4-P
REGENERATION
Sugars
Fructose 6-P
Fructose 1,6-2P
DHAP
REDUCTION
ATP (x6)
ADP (x6)
GAP (x5)
GAP (x6)
GAP (x1)
NADPH (x6)
NADP+ (x6)
Pi (x6)
Calvin cycle
Chloroplast, stroma
Chloroplast
Ribulose 1,5-P2
Glutamine
Calvin cycle
CO2
O2
2Fdxred
2Fdxox
3-Phospho-glycerate
2 Glutamate
ADP
ATP
2-Phosphoglycolate
Glycerate
Glycolate
Glutamate
O2
H2O2
NAD+
NADH
Glyoxylate
H2O + 1/2O2
NH3
Hydroxypyruvate
Peroxisome
Glycine
NAD+ NADH
CO2
NH3
Methylene H4-folate
Glycine
Serine
Mitochondrion
Photorespiration
-5
-2.5
0
2.5
5

Fig. 20.

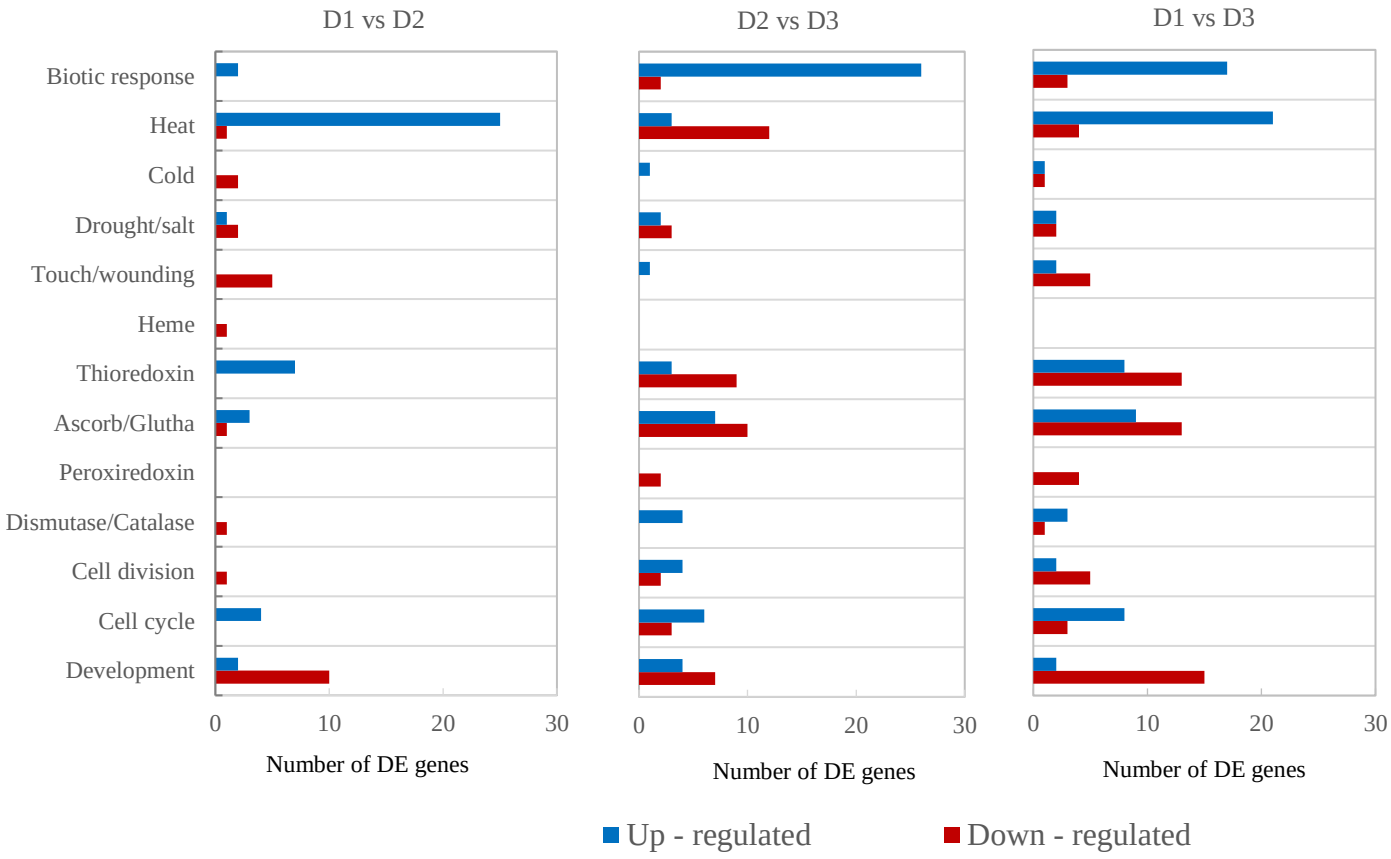

D1 vs D2
D2 vs D3
D1 vs D3
Biotic response
Heat
Cold
Drought/salt
Touch/wounding
Heme
Thioredoxin
Ascorb/Glutha
Peroxiredoxin
Dismutase/Catalase
Cell division
Cell cycle
Development
0
10
20
30
Number of DE genes
Up - regulated
Down - regulated

Fig. 21.

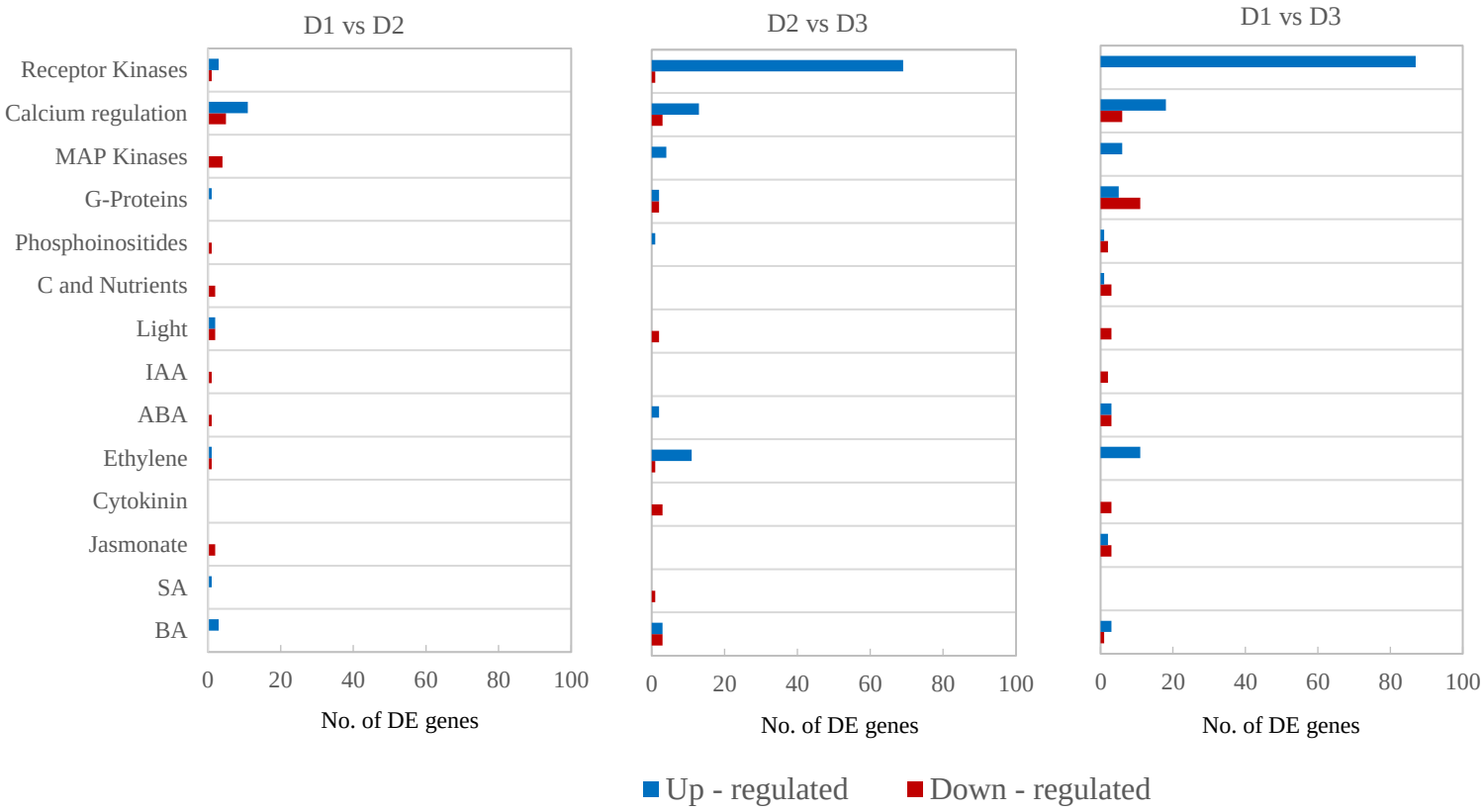

D1 vs D2
D2 vs D3
D1 vs D3
Receptor Kinases
Calcium regulation
MAP Kinases
G-Proteins
Phosphoinositides
C and Nutrients
Light
IAA
ABA
Ethylene
Cytokinin
Jasmonate
SA
BA
0
20
40
60
80
100
No. of DE genes
Up - regulated
Down - regulated

Fig. 22.

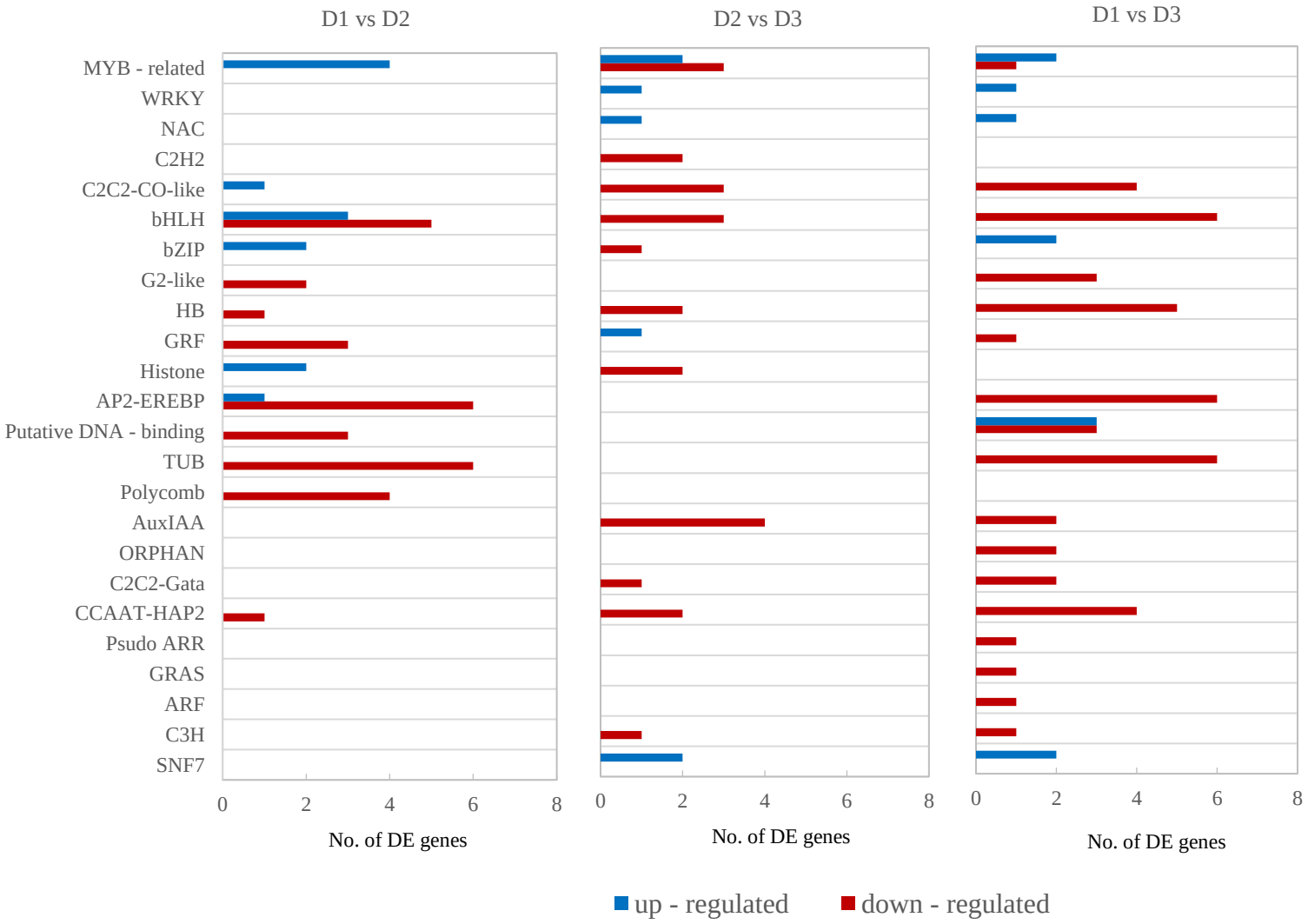

D1 vs D2
D2 vs D3
D1 vs D3
MYB - related
WRKY
NAC
C2H2
C2C2-CO-like
bHLH
bZIP
G2-like
HB
GRF
Histone
AP2-EREBP
Putative DNA - binding
TUB
Polycomb
AuxIAA
ORPHAN
C2C2-Gata
CCAAT-HAP2
Psudo ARR
GRAS
ARF
C3H
SNF7
0
2
4
6
8
No. of DE genes
No. of DE genes
No. of DE genes
up - regulated
down - regulated

Fig. 23.

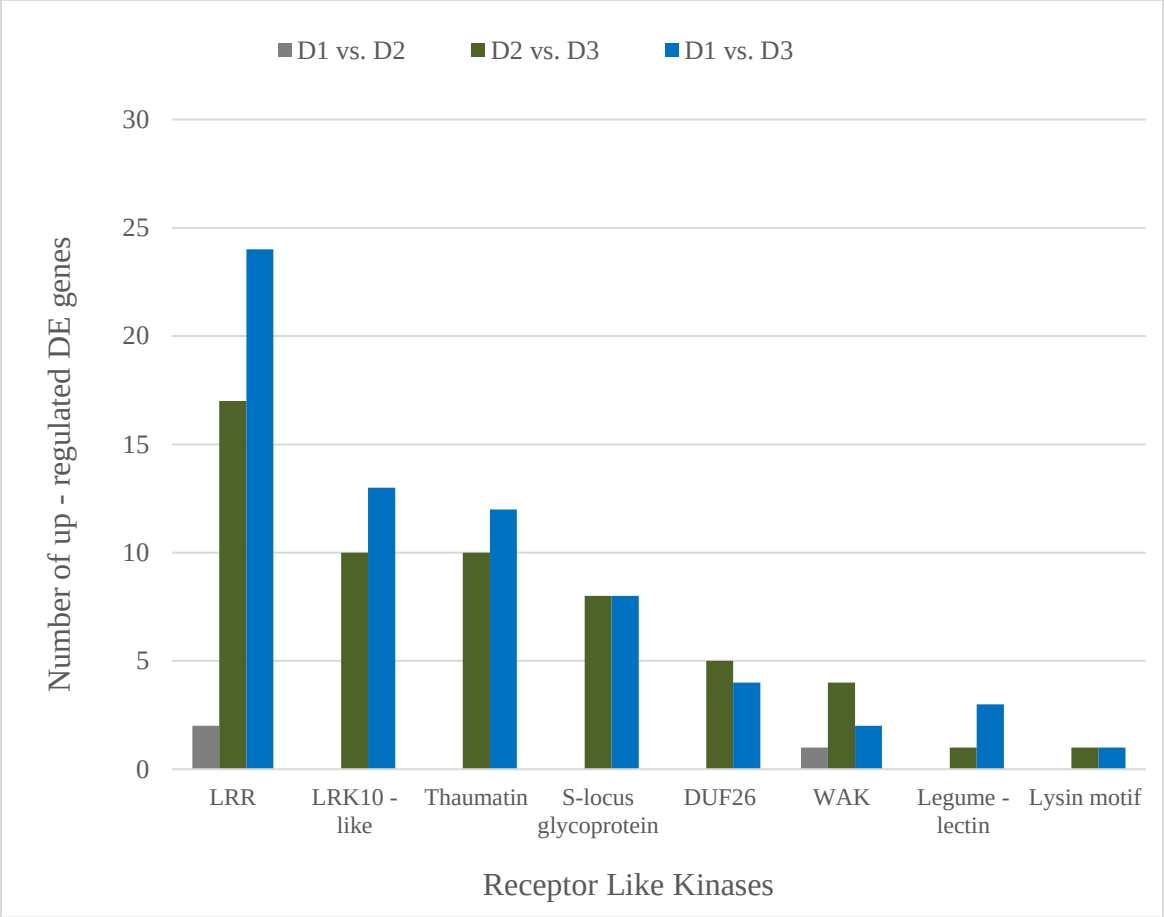

D1 vs. D2
D2 vs. D3
D1 vs. D3
Number of up - regulated DE genes
30
25
20
15
10
5
0
LRR
LRK10 - like
Thaumatin
S-locus glycoprotein
DUF26
WAK
Legume - lectin
Lysin motif
Receptor Like Kinases

Fig. 24.

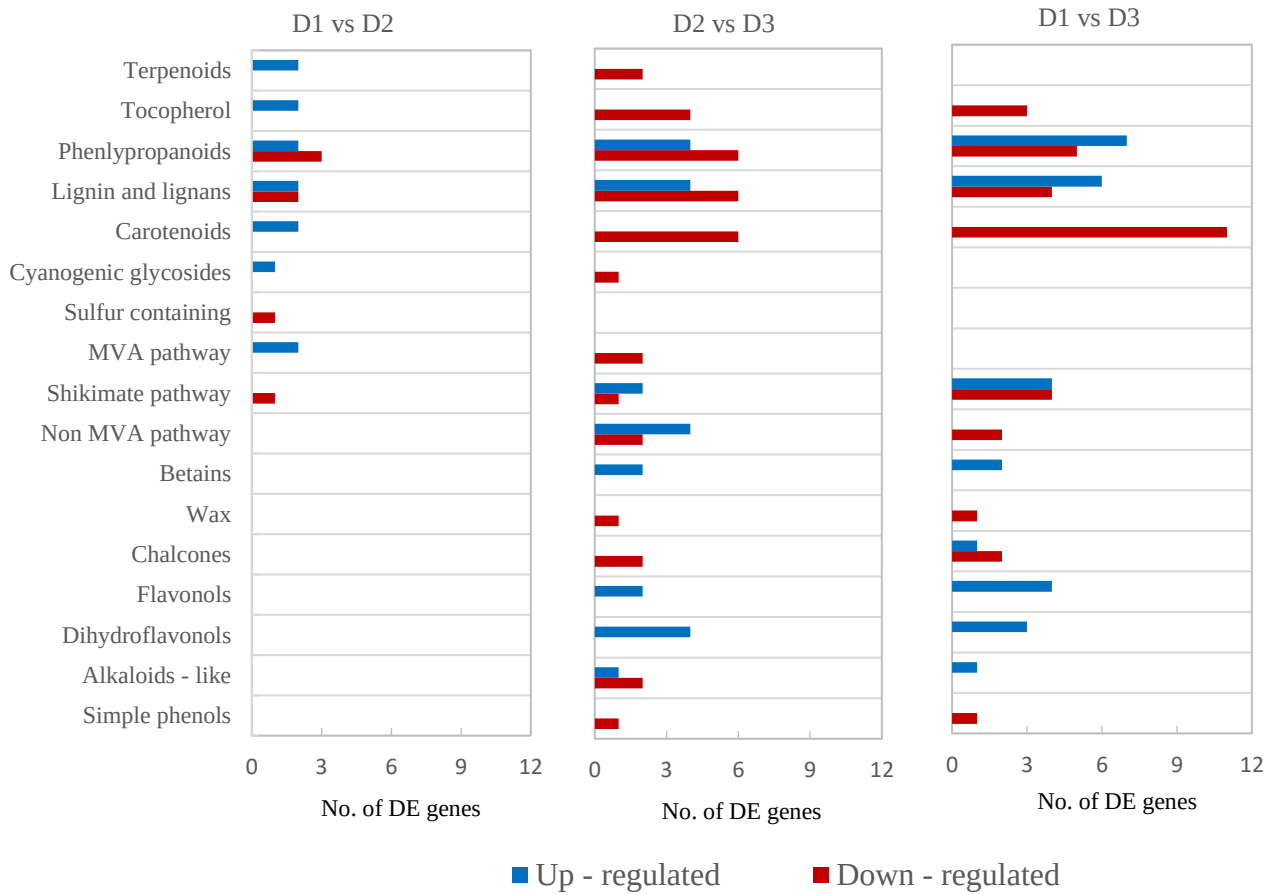

D1 vs D2
D2 vs D3
D1 vs D3
Terpenoids
Tocopherol
Phenlypropanoids
Lignin and lignans
Carotenoids
Cyanogenic glycosides
Sulfur containing
MVA pathway
Shikimate pathway
Non MVA pathway
Betains
Wax
Chalcones
Flavonols
Dihydroflavonols
Alkaloids - like
Simple phenols
0 3 6 9 12
No. of DE genes
Up - regulated
Down - regulated

Fig. 25. A.

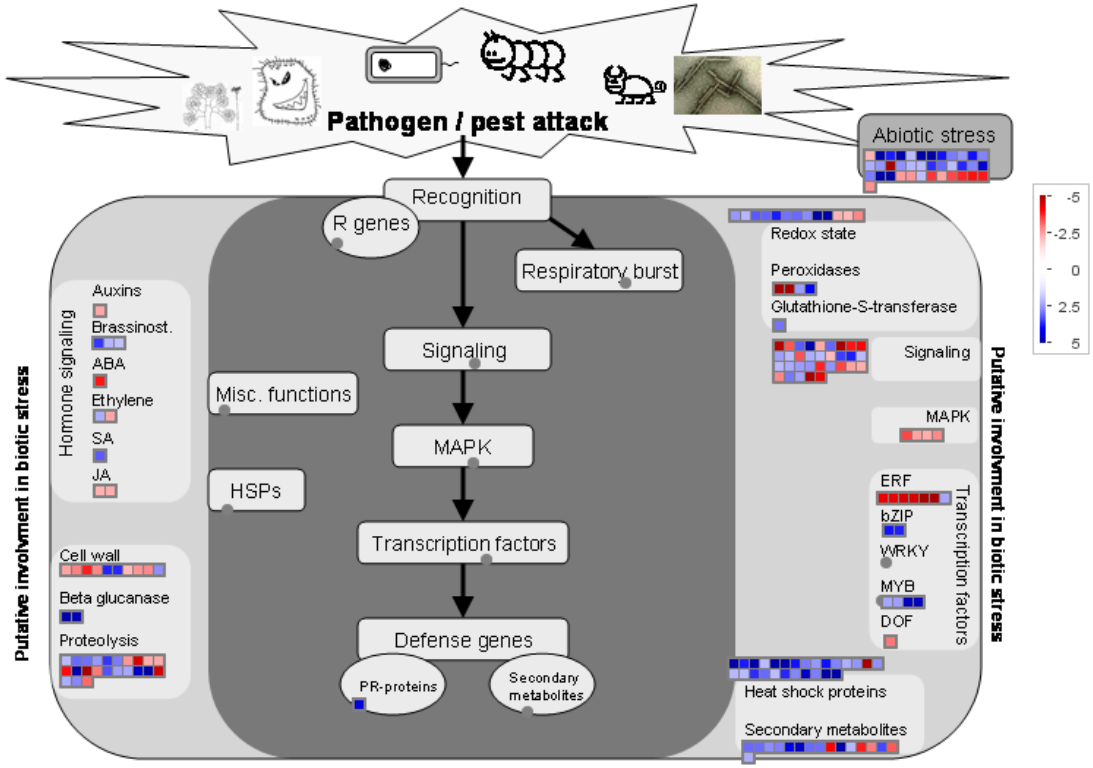

Pathogen / pest attack
Abiotic stress
Recognition
R genes
Respiratory burst
Signaling
Misc. functions
MAPK
HSPs
Transcription factors
Defense genes
PR-proteins
Secondary metabolites
Hormone signaling
Auxins
Brassinost.
ABA
Ethylene
SA
JA
Cell wall
Beta glucanase
Proteolysis
Redox state
Peroxidases
Glutathione-S-transferase
Signaling
MAPK
ERF
bZIP
WRKY
MYB
DOF
Transcription factors
Heat shock proteins
Secondary metabolites
Putative involvment in biotic stress
Putative involvment in biotic stress
-5
-2.5
0
2.5
5

Fig. 25. B.

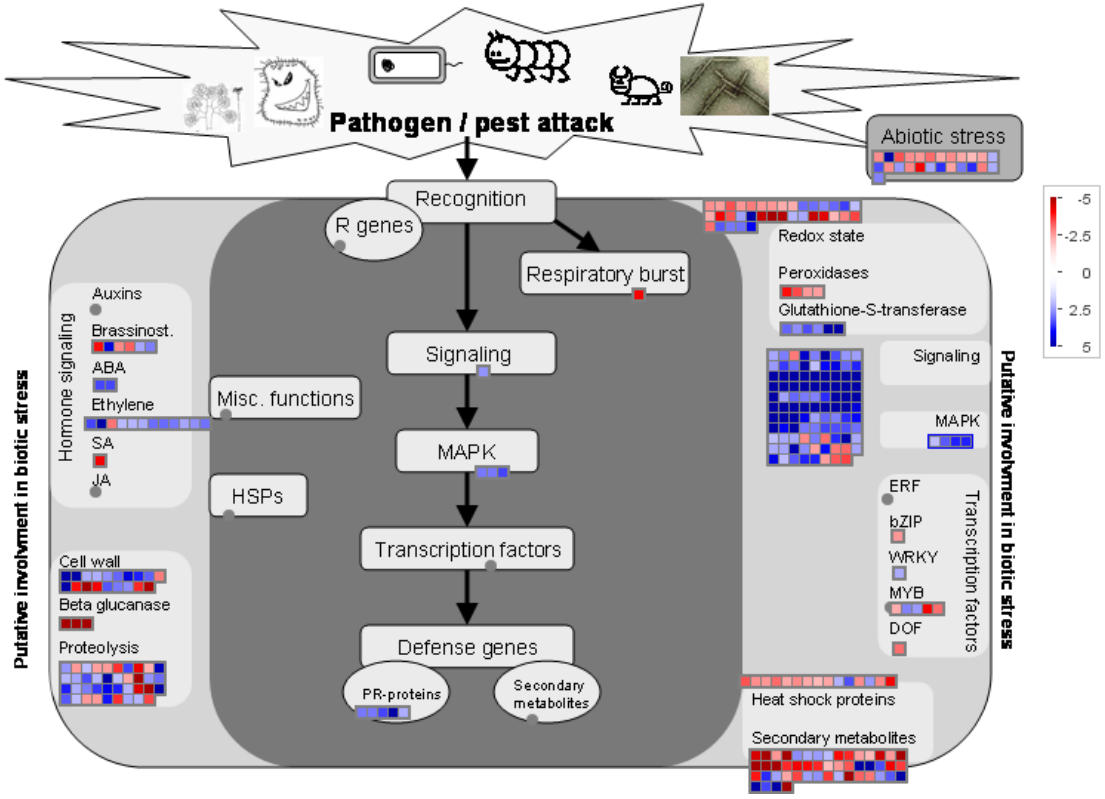

Pathogen / pest attack
Abiotic stress
Recognition
R genes
Respiratory burst
Signaling
MAPK
Transcription factors
Defense genes
PR-proteins
Secondary metabolites
Misc. functions
HSPs
Hormone signaling
Auxins
Brassinost.
ABA
Ethylene
SA
JA
Cell wall
Beta glucanase
Proteolysis
Redox state
Peroxidases
Glutathione-S-transferase
Signaling
MAPK
Transcription factors
ERF
bZIP
WRKY
MYB
DOF
Heat shock proteins
Secondary metabolites
Putative involvment in biotic stress
Putative involvment in biotic stress
-5
-2.5
0
2.5
5

Fig. 25. C.

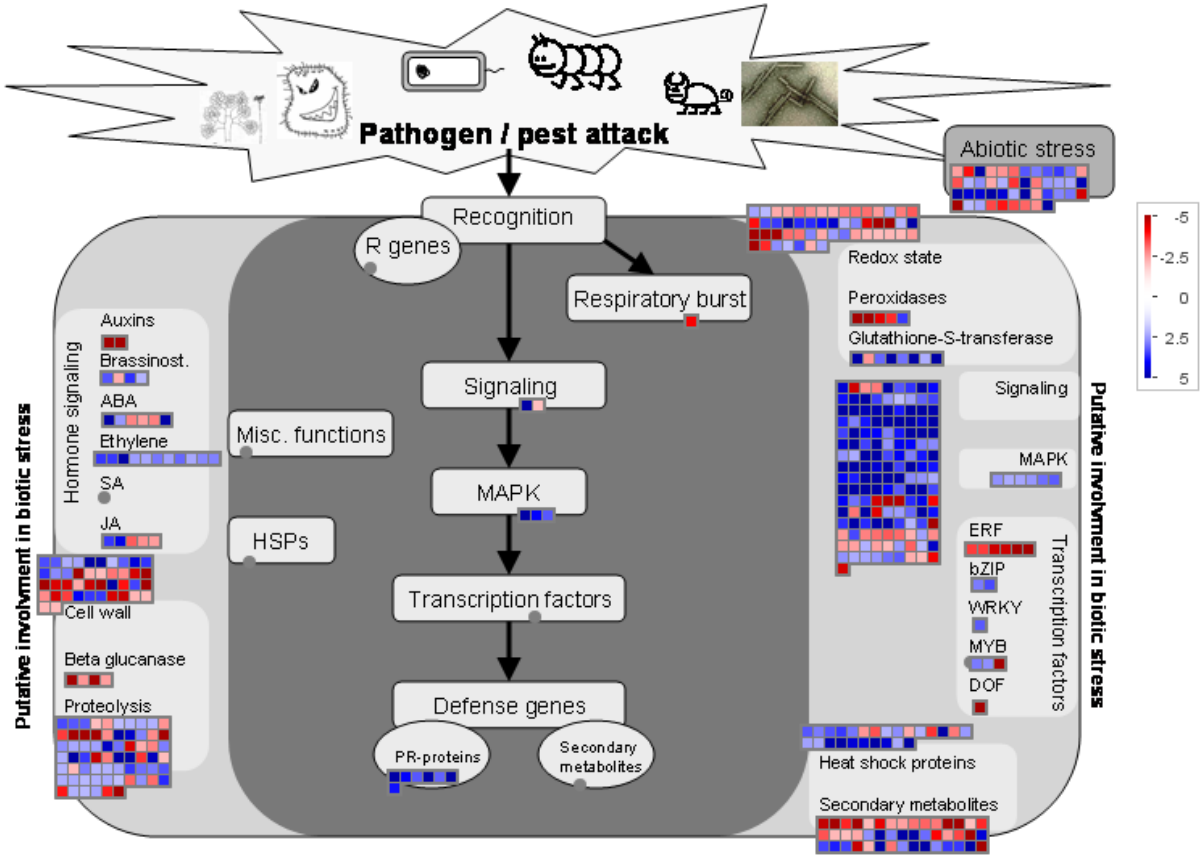
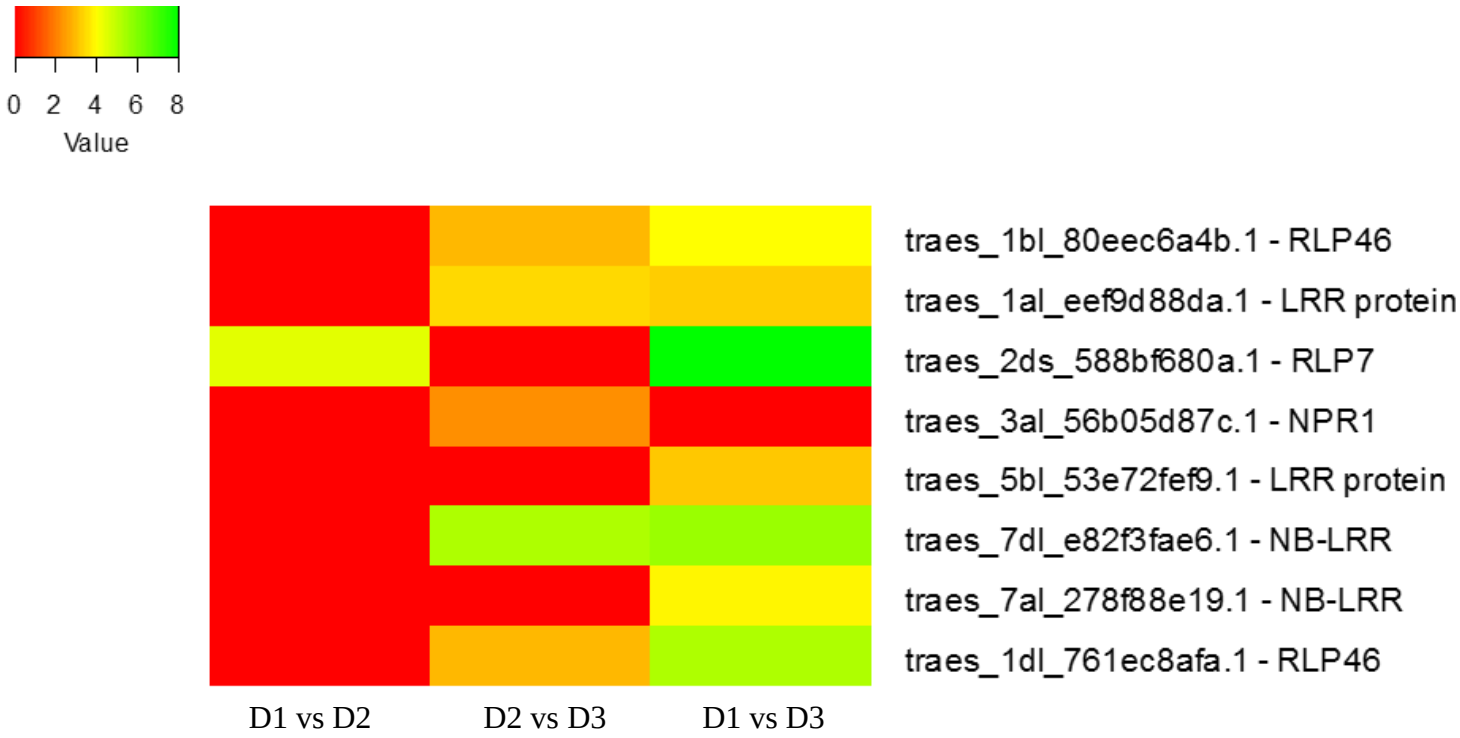

Pathogen / pest attack
Abiotic stress
Recognition
R genes
Respiratory burst
Signaling
Misc. functions
MAPK
HSPs
Transcription factors
Defense genes
PR-proteins
Secondary metabolites
Hormone signaling
Auxins
Brassinost.
ABA
Ethylene
SA
JA
Cell wall
Beta glucanase
Proteolysis
Putative involvment in biotic stress
Redox state
Peroxidases
Glutathione-S-transferase
Signaling
MAPK
ERF
bZIP
WRKY
MYB
DOF
Transcription factors
Heat shock proteins
Secondary metabolites
Putative involvment in biotic stress
-5
-2.5
0
2.5
5

Fig. 26.


0 2 4 6 8
Value
traes_1bl_80eec6a4b.1 - RLP46
traes_1al_eef9d88da.1 - LRR protein
traes_2ds_588bf680a.1 - RLP7
traes_3al_56b05d87c.1 - NPR1
traes_5bl_53e72fef9.1 - LRR protein
traes_7dl_e82f3fae6.1 - NB-LRR
traes_7al_278f88e19.1 - NB-LRR
traes_1dl_761ec8afa.1 - RLP46
D1 vs D2
D2 vs D3
D1 vs D3

CHAPTER 5: Conclusions

Hybrid necrosis is observed in many crops. Epistatic interaction of the divergent loci has been proposed the cause of genetic incomatability resulting to hybrid necrosis (Dobzhansky and Dobzhansky 1937, Muller 1942; Orr 1996). Though the causal genes for specific necrosis in many crops have been identified, underlying cellular and molecular mechanisms are not known. The studies conducted to this dissertation are related to the genetical and physiological process involved during hybrid necrosis of wheat.

Many studies highlights the importance of ROS in programmed cell death of hybrid necrosis (Khanna–Chopra et al., 1998; Mino et al., 2002; Sharma et al., 2003; Mizuno et al., 2010). The first study is intended to figure out whether the ROS is the signaling molecule in hybrid necrosis. Necrotic hybrid leaves generated similar or lower level of H_2O_2 production as that of parent leaves. This suggests that H_2O_2 is not the signal molecule for the programmed cell death in wheat hybrid necrosis. Neither we did measure the other ROS forms i.e., superoxide anion (O_2^-) or hydroxyl (OH^-) nor any antioxidant enzymes. So, the result obtained from the sole H_2O_2 could not be generalized the ROS relation to programmed cell death. In overall, XDH suppression by allopurinol did not reduce the ROS generation and also the necrosis development was not affected. This suggests that the XDH is not the main source of ROS generation in wheat. However, XDH suppressed plants resulted the lower plant height and lower grain yield in one of the

greenhouse study. As the XDH is crucial for fully functional purine catabolism and there by nitrogen re-assimilation, loss of function of XDH could be attributed on such reduced growth and yield. Depriving host nitrogen through the loss of function of XDH by chemical application of allopurinol was considered in the change of susceptible reaction to the hypersensitive type in leaf rust of wheat (Adam et al., 2000).

In temperature tests conducted in growth chambers, plants grown at lower temperature ($15^{O}C > 23^{O}C$) has profound development of necrosis, and necrosis did not appear on plants grown at $30^{O}C$. In some crops, higher temperature role of detoxification of ROS has been proposed for the alleviating the necrosis in tomato hybrid plants (Mino et al., 2002), though that case could not apply in our experiment as we did not observe higher H_2O_2 generation even when the plants went through the normal necrosis process in green house. As the higher temperature role has been reported in inhibiting plant immunity genes (Traw and Bergelson, 2010; Alcazar and Parker, 2011; Hua, 2013), *Ne1* and/or *Ne2* genes expression in intraspecific wheat hybrid necrosis also affected by temperature.

The second study on molecular mapping and characterization of hybrid necrotic genes revealed that the moderate necrosis inducing alleles involved in epistatic interaction have strong effect in phenotypes of the plant. Plants having the genotype *Ne1Ne1 Ne2Ne2* had the most severe necrosis and died earliest, revealing the dosage effect of *Ne1* and *Ne2* alleles. Both the necrosis inducing alleles identified in our study

were characterized as moderate necrosis inducing alleles (Hermsen, 1963a; Chu et al., 2006). *Ne1* gene residing on 5B chromosome contributed 29.3 % of the phenotypic variances and *Ne2* gene residing on 2B govern the 35% of the phenotypic variances and all together they had 94.6% of the phenotypic effects in plants. Out of the 14 polymorphic markers, three of the SSR markers were close to the *Ne2* gene on 2B chromosome. Among the three close markers, Xbarc7, Xbarc13 and Xwmc344 were at a distance of 3 cM, 4 cM and 6 cM from the *Ne2* gene on 2B chromosome. Among the eight polymorphic markers identified on 5B chromosome, Xgwm639 marker was at a distance of 11 cM from the *Ne1* gene on 5B chromosome.

RNA-seq transcriptome analysis as presented in chapter 4 revealed the changes in various physiological process due to hybrid necrosis. Though the change in gene expression began even before the visible symptoms in the leaf, sharp activation in in the genetic expression were revealed with the beginning of necrosis to when the half of leaf became necrotic. This suggests, the rapid change in metabolisms and cellular process and regulation due to hybrid necrosis. The down-regulation of photosynthesis, carbohydrate and amino acid metabolisms due to necrosis caused the decrease in synthesis and consumption of energy, and amino acid. Defense signaling, and biotic and abiotic stress related genes revealed the most up-regulated patterns throughout the growth of necrosis. Our study shows the activation of genes encoding for the PRRs (LRR-RLKs and RLPs responsible for pattern recognition receptors, activation of NB-LRR proteins). The

assignments of expressed genes based on gene ontology revealed the very high activation of polyamine oxidase along with the activation of several antioxidant enzymes. This strongly suggests that there would have higher generation of ROS, and polyamine oxide could be the main source of ROS generation in hybrid necrosis (Yoda et al., 2003, 2006). Along with, there were activation of genes encoding for the CRT3, CRT1, calcium dependent protein kinases (CPK3, CPK7), calmodulins (CAM5, CAM16) and CNX1. Activation of such Ca^{2+} sensors and transporter suggests there was increase in Ca^{2+} in cell, triggering the Ca^{2+} signaling (Mahajan and Tuteja, 2007; Cheval et al., 2013). Similarly, there was up-regulation of genes encoding for the defense related MAP kinases (MAP kinase6, and MKK1) (Tena et al., 2011; Meng and Zhang, 2013). At the hormonal level, genes encoding for the ethylene were the most up-regulated ones. Weaving together all these events i.e., activation of PRRs, ROS generation, Ca^{2+} and MAP kinase signaling, activation of ethylene hormonal pathway strongly resembles with the cascades of pathogen resistance downstream responses. Apparent of these cascades in our study support that the autoimmunity as the cause of hybrid necrosis in wheat.

Our study in chapter 2, there was not higher generation of H_2O_2 in F_1 hybrid plants before and during the progression of necrosis. However, the assigned genes from gene ontology in RNA-seq analysis suggest the higher ROS generation during necrosis. Superoxide anion (O_2^-), H_2O_2 and OH^- are the major forms of ROS (Apel and Hirt, 2004; Tripathy and Oelmuller, 2012). In chapter 2, we did neither measure the superoxide (O_2^-),

and OH^- generated in plants nor any antioxidant enzymes. Upon superoxide (O_2^-) generation in plants, SOD catalyzes the conversion of superoxide to H_2O_2 (Apel and Hirt, 2004). Then, generated H_2O_2 is detoxified by the antioxidant enzymes such as i.e., catalase, the ascorbate peroxidase and glutathione S transferase (Apel and Hirt, Sharma et al., 2003; Tripathy and Oelmuller, 2012). One reason of not detecting the higher H_2O_2 in leaf tissue could be due to not enough activation of superoxide dismutase to convert the superoxide to H_2O_2. Other reason could be due to very high activation of H_2O_2 detoxifying enzymes like catalase, ascorbate peroxidase, and glutathione transferase.

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