HORMONAL AND GENETIC BASIS OF HEAT RESPONSE IN FIELD PEA (*PISUM SATIVUM* L.)

A Thesis Submitted to

the College of Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the Department of Plant Sciences

University of Saskatchewan

Saskatoon, Saskatchewan

By

Shaoming Huang

PERMISSION TO USE

In presenting this thesis in partial fulfilment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Plant Sciences

51 Campus Drive

University of Saskatchewan

Saskatoon, Saskatchewan, S7N 5A8 Canada

OR

Dean

College of Graduate and Postdoctoral Studies

University of Saskatchewan

116 Thorvaldson Building, 110 Science Place

Saskatoon, Saskatchewan S7N 5C9 Canada

ABSTRACT

Field pea (*Pisum sativum* L.), a cool-season legume crop, is known for poor heat tolerance. Over the last decade, progress has been made in the understanding of the physiological damage of pea plants caused by heat stress (HS), however, knowledge of the hormonal and genetic basis of heat stress response (HSR) is still scarce. This thesis was focused on the characterization of abscisic acid (ABA) metabolism and transcriptome response among pea varieties contrasting in heat tolerance.

In Study I, two heat tolerant pea varieties, CDC Meadow and PR11-2, and two sensitive varieties, Nitouche and PR11-90, were evaluated, whose heat tolerance were previously characterized in field trials. Plants of individual varieties were heat stressed for 3 h, 6 h, 12 h or 24 h at 38°C before pollination. RNA extracted from anthers and stipules on the same flowering node were sampled for transcriptional profiling of two pea heat shock protein (HSP) genes, *PsHSP18.1* and *PsHSP71.2*. Additional stipules were sampled for the quantification of ABA concentration and its five key catabolites from the four major ABA catabolic pathways by liquid chromatography-multiple reaction monitoring mass spectrometry. Both pea HSP genes and ABA metabolism responded rapidly after 3 h at HS. However, *PsHSP18.*1 and *PsHSP71.2* had similar induction levels between heat tolerant and susceptible varieties, suggesting the function of these two genes is conserved in heat response of pea. Heat tolerant varieties had a higher ABA synthesis and turnover rate at 3 h HS, than their respective heat susceptible counterparts.

In Study II, heat tolerant variety, PR11-2, and heat susceptible variety, PR11-90, were selected from Study I to characterize the differential transcription at 3 h HS via RNA-Seq technology. The widely grown and moderately heat tolerant variety, CDC Amarillo, was included as a check. Differentially expressed genes (DEGs) were identified at log2 |fold change (FC)| ≥ 2 between HS and control temperature. The three varieties shared 588 DEGs which were up-regulated and 220 genes which were down-regulated in anthers when subjected to HS. In stipules, 463 upregulated genes and 416 downregulated genes were consistent among varieties. The above heat-induced genes of stipules and anthers were related to several biological processes, i.e., response to heat, protein folding, and DNA templated transcription. Ten gene ontology (GO) terms were over-represented in the consistently down-regulated DEGs of the two organs, and these terms were mainly related to cell wall macromolecule metabolism, lipid

transport, lipid localization, and lipid metabolic processes. GO enrichment analysis on distinct DEGs of individual pea varieties suggested that heat affected biological processes were dynamic, and variety distinct responses characterize the heat tolerance variation among pea varieties at the transcriptional level. Several biological processes, e.g., cellular response to DNA damage stimulus in stipule and electron transport chain in anther, that were only observed in heat induced PR11-2 and CDC Amarillo, and their relevance to field pea heat tolerance is worth further validation.

To validate the above transcriptional variation at HS, 39 recombinant inbred lines (RILs) were made from the cross of PR11-2 and CDC Amarillo in Study III, and they were tested in field trials in 2020 and 2021, to investigate the genetic loci associated with heat responsive traits relating to flowering and yield components. In total, four consistent loci were identified to be associated with heat responsive traits over multiple site-years, which were a QTL for days to flowering at chromosome 7, a QTL for pod number at chromosome 2, and one each QTL for reproductive node number and days to maturity at chromosome 5. Notedly, eight genes (5g161560, 5g165160, 5g171400, 5g198960, 7g051680, 7g091560, 7g091680 and 7g093240) within the aforementioned QTLs were differentially expressed between PR11-2 and CDC Amarillo in Study II. As a result, these eight genes were proposed to contribute to the superior heat tolerance of PR11-2 over CDC Amarillo. Collectively, my thesis expands the current understanding of pea heat response at the hormonal, transcriptional and genetic levels.

ACKNOWLEDGEMENTS

Although my emotions at writing this acknowledgement are far beyond my words, I would firstly appreciate my supervisor, Dr. Tom Warkentin, for giving me this valuable opportunity to carry out this PhD thesis, in the subjects of my true interest. Without his consistent patience, encouragement, advice, positivity and hard training, I could not imagine I could complete such a complicated thesis work so smoothly. Besides, he always encourages me not to limit myself and have a broad vision in perceiving science and life.

My timely progress is also credited to the diligence of all my other PhD committee members, Dr. Rosalind Bueckert, Dr. Bunyamin Tar'an, Dr. Art Davis, Dr. Yuguang Bai. They have given countless advice in crop physiology, botany, plant biology, ecology, breeding, genetics and genomics, which guides my research work on a good track. I would like to express my special gratitude to Dr. Peter Pauls from University of Guelph, for generously and timely agreeing to be the external examiner of my thesis, and his subsequent devotion and suggestion in revising my thesis work.

Besides, I would like to thank Dr. Kishore Gali for his helps in various aspects of my thesis work, and many thanks to other research colleagues in pea/soybean group, as well as the whole pulse breeding field crew for their unconditional supports. I appreciate the TA opportunities that Mrs. Krista Wilde, Dr. Pierre Hucl and Dr. Kristin Bett have offered to me in their courses. Lastly, this work has been financially supported by the Saskatchewan Agriculture Development Fund, Saskatchewan Pulse Crop Development Board, and Western Grains Research Foundation.

Lastly, my deep appreciation and love is to my wife, Yinyin Zou, for her love, care, support, patience and encouragement. Crediting to the consistent understanding and support from parents of both sides, I can work onto my research with fully attentions.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABA abscisic acid

ABA-GE abscisic acid-glucose ester

ADF acid-detergent fiber

ATI abiotic tolerance index

CAD cinnamyl alcohol dehydrogenase

CDC crop development centre

cM centiMorgan

CT cycle threshold

CTD canopy temperature depression

DEG differentially expressed gene

DPA dihydrophaseic acid

DTF days to flowering

DTM days to maturity

EE ether extract

ETC electron transport chain

FC fold change

GHG greenhouse gas

GMP geometric mean productivity

GO gene ontology

HS heat stress

HSC heat shock cognate

HSF heat shock transcription factor

HSP heat shock protein

HSR heat stress response

HT high temperature

LAC laccase

LD lodging

LG linkage group

LTP lipid transfer protein

NDF neutral detergent fiber

PA phaseic acid

PME pectin methyl esterase

PN pod number

QTL quantitative trait locus

RIL recombinant inbred line

RNN reproductive node number

RO reproductive organ

sHSP small heat shock protein

SNP single nucleotide polymorphism

SSI stress susceptibility index

STI stress tolerance index

UHPLC-SRM MS ultra-high performance liquid chromatography-selected reaction monitoring mass spectrometry

YSI yield stability index

CHAPTER 1. INTRODUCTION

Field pea (*Pisum sativum* L.) belongs to the cool season *Leguminosae* family, and it is known as a heat susceptible crop. When temperature is beyond the upper-temperature threshold of a plant's normal growth and development, heat stress (HS) starts to damage the plant. Based on the intensity and duration of HS, it is characterized as chronic or acute, and thus has a different detrimental effect on the plant. During the last decade, numerous studies have been conducted on the impact of HS on pea plants, with particular attention to characterizing physiological damage of HS on pea's reproductive organs. When pea plants at anthesis were exposed to 36/18 °C day/night for seven days in a growth chamber, the pollen germination percentage, pollen tube length, pod length, and the seed-ovule ratio dropped significantly compared to peas grown at control temperature condition of 24/18 °C (Jiang et al., 2015). HS also negatively affected anther dehiscence in pea plants of different genotypes (Jiang et al., 2019).

Understanding of the regulatory mechanisms of pea HS response is not yet available. In a plant's HS transcriptional regulation, the best known is the identification of heat shock transcription factors (HSFs) and heat shock protein (HSP) genes (Ohama et al., 2017). HSPs act as molecular chaperones to facilitate folding of other functional proteins and prevent them from denaturation and aggregation during exposure to HS. In vegetable pea, several HSPs in the sub-families of sHSP and HSP 70 were previously characterized, among which PsHSP 17.9, 18.1 and 71.2 were significantly induced under HS (DeRocher and Vierling., 1995; DeRocher et al., 1991; Srikanthbabu et al., 2002). It is worthwhile to validate the role of these HSP genes in the HS response (HSR) of field pea. What's more, genes involved in the HS transcriptional network in model crops were not limited to genes encoding HSF and HSP, and heat responsive genes could expand to those genes relating to plant hormone biosynthesis and signaling, calcium and sugar signaling, primary and secondary metabolism (González-Schain et al., 2016; Liu et al., 2015). In lentil, genes in cell wall development and maintenance, as well as secondary metabolism, also played vital roles (Singh et al., 2019). To date, no transcriptome-wide mapping of pea response

to HS has been conducted, but this method was utilized in the discovery of responsive genes in field pea seed aging (Chen et al., 2013), root nodulation (Alves-Carvalho et al., 2015) and most recently in water-logging stress studies (Henriet et al., 2019; Zaman et al., 2019).

Hormonal regulation is also important in the regulatory network of a plant's HSR. In field pea and other legumes, auxins and ethylene were documented to be involved in HS response during plant reproduction from pollen development to seed maturation (Ozga et al., 2017). In field pea, Abeysingha (2015) found that in the growth chamber, a single application of auxin or its analogue during flowering initiation could alleviate the detrimental HS effect at 35°C for 6 hours per day for 4 days on seed yield to some extent. HS also differentially modified ethylene biosynthesis and signaling among different pea floral and fruit tissues, thereby impairing their growth to various extents (Savada et al., 2017). Genes of abscisic acid (ABA) biosynthesis in Arabidopsis (e.g., *ABA 1*, *ABA 2*, *ABA 4*, *AAO 3*, *NCED 3)* were differentially expressed (up- or down-regulated) in an organ-specific manner among leaves on the flowering nodes, inflorescence meristem, and developing silique (Baron et al., 2012). However, the information on ABA response in HS is lacking in pea.

Traits linked to yield and heat tolerance are typically quantitative and are controlled by multi-gene action (Huang et al., 2017; Tafesse et al., 2020). To gain knowledge on the quantitative traits genetically, a useful method is to build a genetic linkage map, and thereby characterize corresponding quantitative trait loci (QTL) via the integration of phenotypic data. Linkage maps in organisms are genetic maps showing the position and relative genetic distances between specific markers along chromosomes. Mapping populations segregating for heat tolerance are still limited in pulse crops, but have been drawing increasing attention over the last five years. Paul et al. (2018) reported the first genetic mapping of QTLs related to heat tolerance in chickpea, in a study based on 200+ RILs from the cross between the heat tolerant cultivar ICC 15614 and the heat sensitive cultivar ICC 4567. Four consistent QTLs were identified on linkage group (LG) V and VI for yield traits under HS environments, i.e., late seeding trials. An individual consistent QTL was identified for seedling survival and pod set of lentil separately at HS environments under a hydroponic assay, which explained 12% and 9% phenotypic variance of seedling survival and pod set, respectively (Singh et al., 2017). In field pea, Huang et al. (2017) identified ten consistent QTLs associated with flowering and yield component traits, in

particular, a QTL for days to flowering on LG VIb consistent across four field trials varying in temperature stresses.

Based on the studies mentioned above, I hypothesized:

1) Pea HSP genes and ABA homeostasis would respond rapidly in HS and the response would be different between heat tolerant and heat susceptible varieties (Chapter 3).
2) By comparing the pea transcriptomes between HS and control temperature, additional heat responsive genes including heat transcription factors would be characterized; furthermore, comparative analysis of heat responsive genes among pea varieties with contrasting heat tolerance would help in understanding pea heat tolerance at the transcriptional level (Chapter 4).
3) Heat responsive traits e.g., flowering and yield component traits are quantitative traits whose genetic loci could be characterized via genetic linkage mapping (Chapter 5).

CHAPTER 2. LITERATURE REVIEW

2.1 Global pea production

Field pea (*Pisum sativum* L.) is an annual herbaceous plant, and as a cool season crop, it has been widely grown across various temperate zones around the world. It belongs to the Leguminosae family, because it has edible seeds with high protein and starch and relatively low lipid content. It is also known as a pulse crop, and in this category, there are other major pulses, e.g., lentil, chickpea, dry bean and faba bean. Different to 'green pea' or 'vegetable pea' that are mainly consumed freshly or as a canned vegetable; field pea or 'dry pea' is consumed as dry, shelled grain into diets of both humans and livestock, or fractionated into protein-rich and starch-rich components and sold as ingredients into food and industrial markets.

The domestication of field pea is documented to have started as early as 9,000 BC in present-day southern Turkey and northern Syria, making it one of the earliest crops ever cultivated. Subsequently, the production of pea expanded into Europe and later North America. According to the most recent FAO STAT data from 2015-2019, field pea worldwide cultivation has increased to 7-8 million ha growing area annually, and its yearly production is at 12-16 million tonnes. The average global production from 2015-2019 for dry pea is second only to dry bean among pulse crops. During this five-year period, the top five pea producing countries are Canada, Russia, China, United States, and India (FAOSTAT, 2021).

Farmers in Canada began to plant field pea about 100 years ago on a very limited area. The production began to increase, and has been growing consistently since World War II. As of 2020, the Canadian seeded area of field pea reached 1.7 million ha from 59,500 ha in 1981, and its production increased to 4.5 million tonnes from 110,500 tonnes in 1981 (Saskatchewan Specialty Crop Report, 2020). Canada is the leading producer of pea in the world accounting for approximately 1/3 of global production. The pea production mainly takes place in the three prairie provinces, i.e., Saskatchewan, Alberta and Manitoba, and Saskatchewan contributes more

than half. In 2020, Saskatchewan produced 55% of Canada's dry pea (Saskatchewan Specialty Crop Report, 2020).

2.2 Heat stress

Heat stress (HS) refers to the detrimental effects of temperatures beyond the upper-temperature threshold of a plant's normal growth and development. Based on the intensity and duration of HS, it is characterized as chronic or acute. Chronic HS means a relatively long period of mild stress, where the stress temperature ranges from 5-10°C above optimal temperature of the plant's life cycle. On the contrary, acute HS is a more extreme temperature environment over a shorter period. The prevalence and severity of the two stresses vary from region to region. In the spring-sown field pea areas of western Canada, acute HS would cause more damage than chronic HS, especially consecutive hot days during pea's flowering stage.

2.2.1 Global warming and yield reduction due to elevated temperature

Since the industrial revolution, humans have burned fossil fuels and loaded the atmosphere with greenhouse gases (GHGs) at an unprecedented rate, and climate change has become a global problem causing widespread effects on human and natural systems, where global warming is one of most evident phenomena. Since the late 19^{th} century, the annual air temperature on the earth's land has increased by 1.6°C, and is projected to increase another 0.5-1.5°C between 2030 and 2052 according to current greenhouse gas emission rates (Hoegh-Guldberg et al., 2018). HS has become a major abiotic constraint threatening agricultural production around the world. For instance, Lobell and Field (2007) reported that the yield of maize decreased by 8.3% in response to every 1°C rise in temperature during the period 1961-2002 on the global scale. In pulse crops, chickpea in northwest India had a yield loss of 53 kg ha^{-1} for every 1°C increase in seasonal temperature (Kalra et al., 2008).

Canada has a national population accounting for only 0.5% of the world's population, however, its GHG emission accounts for approximate 2% of the total emissions in the world. Between 1990 and 2019, the yearly national GHG emissions rose by 21.4% or 129 Mt CO_2 equivalent, mainly due to the increase in emissions from oil and gas extraction, as well as transportation (Bush and Lemmen, 2019). As a result, the annual temperature across the country and the national summer temperature warmed by 1.6°C and 1.4°C from 1948 to 2013,

respectively (Environment Canada, 2013 and 2014). Yield of pea varieties in western Canadian regional trials was greatest when summer seasonal mean temperature was below 17.5°C, and declined at greater mean temperatures (Bueckert et al., 2015). When for every 1°C increase in the average daily maximum temperature at flowering stage pea yield declined by 0.3 T per ha. A similar outcome was observed for pea production in the Mediterranean region, where yield declined by 0.6 T as a response to every 1°C increase of average temperature during flowering (Ridge and Pye, 1985).

2.2.2 Heat sensitivity in pea and other pulse crops

The best temperature range of cool-season pulse crops is between 10°C and 30°C. A daily maximum temperature at 25°C is generally considered as the HS threshold level in these crops. In Australia, chickpea appears to have the best heat tolerance among pulse crops, followed by faba bean, lentil and field pea, and their cold tolerance is in the reverse order (Siddique, 1999). The best growing temperature range of Australian field pea is reported to be between 13 to 23°C (Mahoney, 1991). In western Canada, the threshold maximum temperature for yield reduction in the field was approximately 28°C daily maximum at reproductive stage and above 17.5°C mean seasonal daily temperature (Bueckert et al., 2015; Huang et al., 2017). Whereas pea seemed to tolerate a higher temperature under growth chamber conditions, Jiang et al. (2015) reported pollen germination rate was significantly reduced between 33°C and 36°C. The threshold temperatures of other legume species and cereal crops were reviewed by Luo (2011). Although temperature thresholds varied across crop species concerned in this review, reproductive stages were generally more susceptible to extreme temperature than vegetative stages. Wheat was an exception in the paper, and its temperature threshold seemed to increase along with the progression of crop growth and development.

2.2.3 Heat stress on vegetative organs of pea

When air temperature is above the optimum, damage to growth and development of vegetative organs begins. Munier-Jolain et al. (2010) identified that lower leaves of pea plants started senescence after a brief exposure to 30/25°C high temperature (HT) condition. And the damage seemed to be permanent, as the leaves could not recover when plants were returned to normal temperature condition at 20/15°C. Pea leaf physiological functions could also be

impaired by HS. The chlorophyll variable fluorescence, a measure of injury to photosynthesis, declined an average 8% after 3 days at 30/15°C in five pea cultivars compared with control temperature at 20/15°C and in the pea cultivar Alaska (McDonald and Paulsen, 1997). Additionally, the thylakoid activity of chloroplasts in the young leaves was inhibited significantly after exposure to 40°C for only 2.5 minutes. In a field trial, canopy temperature of pea genotypes was also affected to various extents, and semi-leafless pea varieties were better able to maintain cool canopy temperature in contrast to normal leaf type peas (Tafesse et al., 2019).

2.2.4 Detrimental effects of heat stress on reproductive organs and fertility of pea

Depending on the intensity and duration, HS affected plant reproductive development and crop production in different ways (Wahid et al., 2007). Reproductive organs (ROs) did not directly abscind at a mild HS, but the mild stress caused a delayed abortion of ROs at young nodes (Guilioni et al., 1997). Guilioni et al. (2003) identified that the reduced number of seeds pea plants developed when subjected to a mild stress, was due to an impairment of the normal pea plant growth rate during the time window from the beginning of flowering to the beginning of seed filling for the last seed-bearing nodes, which is the critical timing for seed set. They inferred that mild HS in most cases would accelerate flowering, consequently hastening reproduction to occur before the plants could assimilate adequate biomass during the vegetative stage, finally causing the acceleration of normal termination of node production during the plant's life cycle, i.e., heat caused early maturation by stopping additional node production.

Compared to the mild HT stress, damage of a short period of extreme HT at flowering stage can affect yield in a more detrimental and direct manner. The damage depends on the timing and duration of HT. The early flowering stage is more sensitive to HS compared to the later flowering stage, and longer duration of heat exposure caused more detrimental effects on reproductive development (Jiang et al., 2019). The yield loss induced by extreme HT is mainly caused by the abscission of ROs, especially HS induced abortions on flowers and young pods. Jeuffroy et al. (1990) reported that 33/30°C day/night directly led to the abortion of ROs of pea and reduced the seed number per pod. A negative association was further characterized in peas growing at HS and the number of viable flowers, which bears healthy pollens for normal germination (Petkova et al., 2008). This relationship was confirmed in a recent pea growth

chamber study, where Jiang et al. (2015) found that when pea plants at anthesis were exposed to 36/18 °C day/night for seven days in a growth chamber, the pollen germination percentage, pollen tube length, pod length, number of seeds within a pod, and the seed-ovule ratio dropped dramatically compared to pea exposed to normal conditions of 24/18 °C. Moreover, Jiang et al. (2015) indicated that the heat tolerance difference between two pea cultivars, CDC Sage and CDC Golden, might be due to the different lipid composition in their pollen grains. In a parallel study, Lahlali et al. (2014) added that the richness of protein with α-helical structures in the pollen grain of CDC Sage might also help pollen survival under a HT environment. Failure in anther dehiscence was reported in pea when exposed to HT for 7 days (Jiang et al., 2019). Further, male floral organs demonstrated inferior heat tolerance to the female counterpart, as HT dramatically reduced *in vitro* pollen germination rate and pollen-tube growth, but it failed to affect ovule fertilization. Still, ovule sensitivity to HT varied among pea varieties and depended on its relative position within its pod.

Although much less is reported about the effects of HT on female organs, from the published papers the heat damage to female organs was classified into two groups. Firstly, HT could shorten the time of stigma receptivity to pollen, therefore causing asynchrony and failed fertilization. For example, the stigmas in peach (*Prunus persica* L.) lost their ability to support pollen germination on the third day of exposure to 30 °C, and their receptivity was five days shorter compared to normal temperature (Hedhly, 2011). Secondly, HS reduced the total number of ovules and increased ovule abortion.

2.3 Transcriptional profiling of pea response to heat stress

A plant's tolerance to either mild or extreme HS is a complex trait, which involves the interaction of multiple genes. Previous efforts to improve HS tolerance via transgenic methods had largely validated that tolerance was dependant on multiple gene actions. Attempts were made to introgress heat shock protein (HSP) genes from Arabidopsis into crop plants, and the transgenic plants demonstrated improved tolerance under indoor laboratory environments to some extent. However, the benefit of improved heat tolerance failed to result in a sufficient increase of agronomic value under field environments at HT (Fragkostefanakis et al., 2015). Therefore, a global transcriptional profiling might shed more light to the discovery of heat responsive genes and towards breeding for improved heat tolerance.

2.3.1 Transcription profiling methods

The transcriptome is the complete set of transcripts in a cell. Understanding the transcripts and their relative expression is of critical importance to annotate the functional genomics and reveal the molecular constituents at the cellular level. In general, two technologies have been developed to deduce and quantify the transcriptome, i.e., hybridization- or sequence-based approaches (Clark et al., 2002; Wang et al., 2009). DNA hybridization is the reannealing between a single-stranded DNA with another single-stranded DNA from a different source. If the single strand cDNA of interest has sequences that are complementary to the probed DNA strands in the microarray, the two strands will bond tightly and form a double strand DNA hybrid, whose quantity could be further relatively identified via the intensity of fluorescence signal characterized (Clark et al., 2002). RNA-Seq was derived from the more recently developed deep-sequencing technologies, where a population of RNAs of interest were firstly converted into their cDNA library and thereby the transcriptomic cDNA library could be deeply sequenced via next-generation sequencing techniques (Wang et al., 2009). The properties of individual methods are listed in Table 2.1.

Table 2.1. Properties of different transcriptomic methods.

Technology	Microarray	RNA-Seq
Principle	Hybridization	Shotgun sequencing
Resolution	Several to 100 bp	Single base
Throughput	Low to high	High
Reliance on genomic sequence	Yes	No
Background noise	High	Low
Required amount of RNA	High	Low
Cost for large genome sequencing	High	Low

2.3.1.1 Microarray technique

The philosophy of hybridization-based approaches is to hybridize cDNA dyed by fluorescence label with custom-made or commercial high-density oligo microarrays. By the relative quantification of fluorescence signal, the quantitative expression measurements of the corresponding genes can be achieved. The first high-capacity microarray system was developed in *Arabidopsis thaliana*, monitoring the expression of 54 genes in parallel (Schena et al., 1995).

With the development of different array platforms, this method could produce high throughput results at an inexpensive cost, and the method has been widely applied in various crops including pea for biotic and abiotic studies. For instance, 346 out of 16,470 transcripts analyzed were either up- or down-regulated in pea cultivar cv. 'Messire' after inoculation with *Mycosphaerella pinodes* (Fondevilla et al., 2011). Lucau-Danila et al. (2012) produced 4946 transcripts to distinguish chilling and acclimation mechanisms in pea roots and leaves, among which more than 300 transcripts were detected differentially expressed relating to osmolyte protection, photosynthesis, cell wall dynamic architecture, and cell defense mechanism. Still, limitations existed in these methods, e.g., reliance upon the knowledge of a reference genome sequence, high background noise due to cross-hybridization, and difficulty in expression level comparisons among different publications.

The comprehensive expression profiling for HS responsive gene discovery via microarray was limited to the model plant *A. thaliana* and major crops. Swindell et al. (2007) firstly used the Arabidopsis ATH1 Affymetrix microarray platform to profile the transcription patterns of young roots and shoots exposed to various abiotic stresses including heat, drought and cold. HSP 20 family had the highest HS expression response on both roots and shoots, following by HSP 70 and 90 families. The expression differential of HSP 100 family between HS and normal temperature treatment was not significant. In addition to HSP genes, other HS responsive genes were characterized in wheat seedling leaves via the Wheat Genome Array. These putative genes were involved in calcium and sugar pathways, RNA metabolism as well as primary and secondary metabolisms (Qin et al., 2008). Comparative study between barley heat and drought responsive genes revealed that a third of these genes overlapped (Mangelsen et al., 2011). A global transcription profile of canola siliques under HS showed that some heat responsive genes were conserved among plants (Yu et al., 2014).

2.3.1.2 RNA-Seq

Sequence-based approaches to global transcriptome profiling include expressed sequence tags based on Sanger sequencing, and RNA-Seq based on next generation sequencing technologies, for example, Illumina and Roche 454 Life Science (Metzker, 2010). RNA-Seq has been regarded as a more robust and sensitive tool than the microarray approach for transcriptome sequencing. Millions or even billions of reads could be obtained from a single run of sequencing

(Wang et al., 2009). Due to this very high throughput technique, even the expression of low copy transcript genes could be detected in plant tissues. More importantly, RNA-Seq could be conducted irrespective of the availability of a reference genome sequence. In principle, RNA-Seq involves three steps: 1) isolation of a full set of mRNA for the synthesis of a cDNA library, 2) shotgun sequencing of the cDNA library, 3) reads alignment to reference transcripts or to the genome, or *de novo* assembly of the reads when lacking a genomic sequence. RNA-Seq has been used in pea research over the past decade. Franssen et al. (2011) first described large scale transcriptome sequencing with the objective to provide a comprehensive unigene reference set for further analysis in pea. Duarte et al. (2014) used the Roche 454 platform to develop high throughout SNP markers and a high-resolution genetic map syntenous to *Medicago truncatula*. Sudheesh et al. (2015) sequenced 23 cDNA libraries via the Illumina platform for the development of genetic markers, target gene detection on the basis of expression analysis, as well as for a comparative genomics study. In a parallel study by Alves-Carvalho et al. (2015), 20 cDNA libraries, which were derived from various plant tissues at different developmental stages under contrasting nitrogen environments, were sequenced via a similar protocol, and thereby pea orthologs of major nodulation genes identified in model plants were characterized. More recently, RNA-Seq was used in the study of gene response to water stress in pea (Henriet et al., 2019; Zaman et al., 2019). As the pea reference genome is available now (Kreplak et al., 2019), there is a potential to better apply RNA-Seq methodologies for the characterization of responsive genes in various fields of abiotic and biotic stresses in pea.

2.3.2 Transcriptional regulation by heat stress

2.3.2.1 Heat Shock Factors

About 5% of the plant transcriptome is estimated to be up-regulated two-fold or more in response to HS. The presence of a heat-shock element in the promoter is highly linked with the expression induction of many genes that are heat-inducible. Heat shock elements are made of alternating units of pentameric nucleotides (5′-nGAAn-3′) binding to heat shock factors (HSFs). HSFs regulate gene expression upon HS (Ohama et al., 2017).

Plant HSFs are more diverse than those from other organisms. The best characterized HSF gene family in plants was reported in Arabidopsis. The 21 identified HSF genes are categorized into three major classes (HSF A, HSF B and HSF C), among which class HSF A was

most responsible for heat-induced activation of heat shock genes (Nover et al., 2001). HSF A1s are predicted to be the "master regulators" that have the direct role in the activation of the transcriptional network. Knockdown of HSF A1 genes in Arabidopsis led to a reduced induction of many HS-responsive genes; as a result, plants demonstrated HS sensitive phenotypes (Yoshida et al., 2011). The expression levels of important HS-responsive transcription factors are considered to be directly regulated by HSF A1. These transcription factors include, but are not limited to, HSF A2, HSF A7a, HSF Bs and multiprotein bridging factor 1C. HSF A3 is an important HS-responsive TF, because knockout or knockdown mutation of HSF A3 results in reduced expression of putative target HSP genes during HS (Yoshida et al., 2008). The thermo-tolerance conferred by Arabidopsis HSF A1d was further confirmed in a recent study in pea (Shah et al., 2020). A genome-wide expression analysis in tomato anthers revealed that many HSFs were regulated in anthers at the pollen mother cell stage upon HS, and the authors concluded that HSF was the critical factor in the HS response in pollen development (Giorno et al., 2013). Q-PCR expression analysis of chickpea HSFs under heat stress at 15 days old seedling and pod development stages showed that *CarHSF A2, A6,* and *B2* were constitutively up-regulated at both plant development stages indicating their importance in the regulatory network relative to HS (Chidambaranathan et al., 2018).

2.3.2.2 Heat Shock Proteins

In plant cellular defense against HS, the induction of HSPs is one of the major responses. HSPs act as molecular chaperones which are proteins that facilitate folding of other functional proteins especially at the secondary and tertiary structure during non-stress periods and prevent them from denaturation and aggregation during exposure to HS. Depending on the molecular size, HSPs are divided into five conserved classes: (1) small HSPs (sHSPs) whose molecular weights ranged between 10 and 40 kDa; (2) HSP 60 kDa, i.e., HSP 60; (3) HSP 70; (4) HSP 90; and HSP 100. Although HSPs were first reported in HS studies, later they were also found to be involved in other abiotic stress responses including drought and salinity. Based on their expression patterns, HSPs within each family can be also classified into three sub-categories as reported in PsHSP 70 family: 1) only heat-induced, 2) expressed constitutively, and 3) expressed constitutively with additional heat induction (DeRocher et al., 1991).

The sHSPs stand out among the five HSPs families due to their abundance in plants, multiple locations in different cellular compartments and their strong induction by heat. Their expression could rise up to 200-fold under HS (Wang et al, 2004). Generally, sHSPs function as molecular chaperones and protect the substrate proteins against thermal aggregation or denaturation. In six legume species, more than five different classes of sHSPs were detected from plant tissues exposed to HS (Hernandez and Vierling, 1993). In pea, several sHSPs belonging to two classes based on their sequence alignment and immunological cross-reactivity were isolated. PsHSP 17.7, 17.9, 18.1 were located in the cytoplasm, whereas PsHSP 21 and PsHSP 22 were located in chloroplasts and mitochondria, respectively (DeRocher and Vierling, 1991; Lenne et al., 1995). From these reports, we could conclude that they were all involved in establishing cellular thermotolerance to some degree, though the induction of their expression was triggered at different temperatures.

Heat shock protein 70s have also been extensively studied; they are ATP-driven molecular chaperones with an N-terminal ATPase domain and a C-terminal peptide binding domain. Similar to the gene family encoding sHSPs, HSP 70 genes also encode proteins targeted to different cellular compartments, including mitochondria, chloroplast, endoplasmic reticulum, and the cytoplasm, whose expression was detected under both normal and HS environments, and in different plant organs during plant growth. For example, several plant cytosolic HSP 70 genes in rice were constitutively expressed during seed development and maturation, but their levels dramatically dropped within 72 h after the initiation of seed imbibition (Sarkar et al, 2013). As mentioned above, HSP 70s in pea differed in their expression under different temperature environments, inferring functional differences between heat-induced and constitutively expressed HSP 70 homologues (DeRocher and Vierling, 1995).

2.4 Hormone responses of legumes and other model species to heat stress

In addition to transcriptional regulation, plants also respond to HS at the hormonal level. Auxins, abscisic acid (ABA), and ethylene were documented to be involved in HS response during plant reproduction from pollen development to seed maturation (Ozga et al., 2017).

Auxins are essential hormones to regulate a plant's reproductive growth and development. In pea, consistent expression of auxin was found in the pollen grain development and the initiation of stamens (DeMason and Polowick, 2009). In Arabidopsis, the reduced auxin

concentration in the developing anthers was an early HS response (Higashitani, 2013). In field pea, Abeysingha (2015) found that in the growth chamber, a single application of auxin or its analogue during flowering initiation could alleviate the detrimental HS effect at 35°C for 6 hours per day for 4 days on seed yield to some extent. In common bean (*Phaseolus vulgaris* L.), Ofir et al. (1993) found a strong correlation between HS-induced pod abscission and the amount of IAA exported from flowering. Furthermore, a heat tolerant cultivar had a smaller reduction in exported IAA than the heat susceptible cultivars.

On the contrary, abiotic stresses including HS, salinity and drought induced ethylene levels. The induced ethylene level was suggested to aid in reducing growth and protecting against HS-induced oxidative damage, but it could also lead to the death and abscission of different plant organs (Ozga et al., 2017). The ethylene-insensitive mutants *ert1* and *ein2* of *Arabidopsis* are more susceptible to HS than wild types due to induced oxidative stress (Larkindale et al., 2005). Whereas in wheat, 38°C HS at early kernel development substantially induced ethylene production in heat susceptible cultivar, Karl 92, not in heat tolerant cultivar, Halberd (Hays et al., 2007). In pea floral and fruit tissues, ethylene biosynthesis and signaling seemed to be differentially modified by HS (Savada et al., 2017). Two genes in ethylene biosynthesis for converting S-adenosyl-l-methionine to ethylene, i.e., *PsACS* and *PsACO,* had their transcription highly induced at HS in pre-pollinated ovaries but not in pollinated ovaries. The over-expressions of these two genes facilitated the senescence induction in un-pollinated ovaries; as a result, the resource could preferentially move to fertilized ovules and developing seeds for yield compensation at HS.

Compared with the above-mentioned plant hormones, the role of ABA in a pea plant's growth and development at normal temperature and HS is less elucidated. In pea, two low molecular weight (17 and 18 kDa) ABA protein families (ABR 17 and ABR 18) have been isolated and shown to be produced late in seed development (Iturriaga et al., 1994). The constitutive expression of the pea *ABR17* cDNA appeared to play a role in stress tolerance, as three *A. thaliana* transgenic lines with *ABR 17* had better germination when subjected to salt, cold temperature or both (Srivastava et al., 2006). ABA biosynthesis genes in Arabidopsis e.g., *ABA 1*, *ABA 2*, *ABA 4*, *AAO 3*, *NCED 3* were differentially expressed (up- or down-regulated) in an organ-specific manner among stem leaves on the flowering nodes, inflorescence meristem, and developing silique (Baron et al., 2012). Studies in cereal crops revealed the importance of

maintaining ABA catabolism and homeostasis in the adaptation of cold and drought stresses at the reproductive stage (Ji et al., 2011). ABA related study is required in pea to decipher the hormonal regulation in HS.

2.5 Breeding strategies for the improved heat tolerance

2.5.1 Heat stress adaptation mechanisms and possible targeted traits

The adaptive mechanisms dealing with HS can be classified into the three groups, that is, escape mechanisms, avoidance mechanisms and tolerance mechanisms, which were similar to the ecological framework reported in water stress (Bueckert and Clarke, 2013).

Escape mechanisms are generally related to the traits of early maturity. HS can accelerate phenology, early flowering pea cultivars mature earlier, thereby they are able to escape the HS at pod-filling. In breeding cultivars with better adaptation to heat and drought, early flowering and maturity were beneficial heat escape mechanisms and serve as useful criteria for selection for heat resistant cultivars (Hall, 1992). However, research demonstrates a drawback with this escape mechanism which shortens the vegetative period and reduces yield potential in a year with mild or no stress. Evidence in field pea was that a positive correlation between days to flowering and plot yield was observed (Huang et al., 2017).

In avoidance mechanisms, traits of canopy temperature depression, leaf reflectance and stomatal opening, i.e., transpirational cooling, are essentially physiological components. Leaves play a critical role in changing their orientation, morphology, transpiration rate and reflectance, and eventually affect canopy temperature. Tafesse et al. (2019) found that peas with the semi-leafless leaf type and upright plant habit had superior lodging resistance and had better canopy temperature depression in heat and drought field environments than varieties with normal leaf type and lodging plant habit.

Heat tolerance mechanisms of plants can be complex, and three major thermo-tolerance types are categorized, which are basal thermo-tolerance, acquired thermo-tolerance, and thermo-tolerance to extreme HT (Ye et al., 2012). A general heat tolerance is usually achieved by a combination of physiological traits e.g., thermo-stability of cellular membrane, cell wall lipid composition readjustment, and HSP induction and accumulation (Wahid et al, 2007). In addition, the delayed leaf senescence or ‘stay-green’ trait, which results in maintenance of leaf chlorophyll

and photosynthetic capacity, is also considered an important factor for heat tolerance in crop breeding programs (Abdelrahman et al., 2017). Many heat tolerant genotypes exhibit delayed leaf senescence and lower RO sterility when exposed to HS at reproductive stage. The high photosynthetic activity can largely improve ROs viability upon HS, because more carbon-hydrates and assimilates can be synthesized from the relatively active photosynthesis; as a result sufficient energy source can be utilized by ROs for their healthy developments. An increasing number of quantitative trait loci (QTLs) for 'stay-green' have been recently characterized across different crop species, which implies the big potential for the development of its genetic marker to facilitate the selection of heat tolerant genotypes. Also, indeterminate growth habit with a prolonged duration of flowering can be another possible way, because cultivars can recover from a short severe stress and resume flowering.

2.5.2 Marker-assisted selection for heat tolerance

Traits linked to yield and heat tolerance are typically quantitative traits and are controlled by several genes and different genetic mechanisms (Wahid et al, 2007). Numerous studies in crop heat-tolerance are mainly interested in physiological traits, and their accurate assessments are often costly and not high-throughput. With the rapid program in sequencing technique and bioinformatic analysis tool, QTL mapping and subsequent marker assisted selection (MAS) gains an increasing attention, and becomes a promising complement to the conventional breeding approach. MAS allows assessment of numbers, locations, and the magnitude of phenotypic effects and pattern of gene action. Also, MAS allows assessment of many traits of interest, expressed at different developmental stages, at one time.

Linkage maps in organisms are genetic maps showing the position and relative genetic distances between specific markers along chromosomes. The construction of pea linkage maps started in the 1920s via classical two-point crosses using morphological markers. With the advent of PCR, molecular markers such as randomly amplified polymorphic DNA markers, amplified fragment length polymorphism markers, and simple sequence repeats were used to construct linkage maps with increased density. More recently, with the use of next generation sequencing technology, saturated linkage maps (Franssen et al., 2011; Sindhu et al., 2014; Duarte et al., 2014; Huang et al., 2017; Gali et al., 2018) were built using single nucleotide polymorphism (SNP) markers. SNP markers stand out in comparison to other markers due to

their abundancy, their relatively low rates of mutation, their even distribution across genomes, and their relative ease of detection.

Mapping populations segregating for heat tolerance are still limited in pulse crops but have been drawing increasing attention over the last five years. Paul et al. (2018) reported the first genetic mapping of QTLs on heat tolerance in chickpea, whose study was based on 200+ RILs from the cross between heat tolerant cultivar ICC 15614 and heat sensitive cultivar ICC 4567. Four consistent QTLs were identified on linkage group (LG) V and VI for yield traits under HS environments, i.e., late seeding trials. At the similar time, an individual consistent QTL was identified for seedling survival and pod set of lentil at HS environments under a hydroponic assay. However, the identified QTLs seemed to only have minor effects on these two traits as individual QTL only explained 12.1% and 9.2% phenotypic variance for seedling survival and pod set, respectively (Singh et al., 2017).

2.5.3 Status of breeding for heat tolerance in field pea

Recently, a series of research projects have been conducted related to heat tolerance and the identification of its underlying genetic control in pea. Huang et al. (2017) identified ten consistent QTLs associated with flowering and yield component traits, in particular, a QTL for days to flowering on LG VIb consistent across four field trials. Three lines (PR11-2, PR11-88 and PR11-91) were identified as heat tolerant from RIL population PR11, due to their superior yielding performance under both normal and heat stressful environments. PR11-2 was regarded as best heat tolerant RIL with several superior traits including relatively long flowering duration, high pod number per plant, and high grain yield relative to the parents and other RILs. Contrastingly, PR11-90 was considered as the most heat susceptible line in the population.

Evaluation of heat tolerance in pea targeting several vegetative and reproductive traits has been conducted. Tafesse et al. (2019) focused on canopy cooling traits such as CTD and flower abortion in field trials, validated the existence of genetic variability in CTD among 24 genotypes, and elucidated the contribution of leaf wax and pigment to a cooler canopy. Jiang et al. (2020) screened *in vitro* pollen germination and pollen-tube growth over the same 24 genotypes and reported that genetic variability existed for pollen germination. However, in a parallel association mapping study of 92 genotypes, SNPs associated with pollen germination were not detected (Jiang et al., 2017). It is worth noting that novel loci for days to flowering and flowering duration

were identified from this wide pea genetic source compared to the loci detected in the pea RIL population by Huang et al. (2017), implying the complex genetic mechanism for flowering control. Furthermore, 48 candidate genes were characterized for pea heat responsive traits including chlorophyll concentration, photochemical reflectance and reproductive stem length, in a genome-wide association study (Tafesse et al. 2020).

CDC Meadow (Warkentin et al., 2007) demonstrated superior heat tolerance out of the 24 genotypes studied by Tafesse et al. (2019) and Jiang et al. (2020), as it had consistently superior performances in CTD, pod number per plant, and *in vitro* pollen germination, but low ovule abortion and flower abortion. In addition, CDC Meadow is one of the pea accessions in the PAM and GWAS panels (Crop Development Centre pea breeding program) with lowest pod abortion, high yield, and it is the most widely grown pea cultivar in North America over the past five years. Based on these findings, CDC Meadow is suggested as a heat tolerant cultivar in the proposed experiment of transcriptional analysis of HS. Nitouche is used as a heat sensitive cultivar due to its high ovule abortion rate and relatively poor yield performance under HT (Jiang et al., 2019). It is one of the pea accessions with the worst heat tolerance in terms of high pod abortion and low yield in both PAM and GWAS panels over multiple site-years experiment.

CHAPTER 3. COMPARATIVE ANALYSIS OF HEAT STRESS INDUCED ABA AND HEAT SHOCK PROTEIN RESPONSES AMONG PEA VARIETIES

This chapter is under review at Crop Science (submitted March 14, 2022).

'**Huang, S**., Zhang, H., Purves, R.W., Bueckert, R.A., Tar'an, B., and Warkentin, T.D. Comparative analysis of heat stress induced ABA and heat shock protein responses among pea varieties.'

3.1 Abstract

Field pea (*Pisum sativum*) belongs to the cool-season legume family, and it is known for heat susceptibility. Heat shock proteins (HSPs) and abscisic acid (ABA) play important roles in plant heat responses, but have not been extensively studied in pea. In this study, four pea varieties varying in heat tolerance based on field trials were evaluated. Plants were heat stressed for 3 h, 6 h, 12 h or 24 h at 38°C before pollination. RNAs of anthers and stipules on the same flowering node were sampled for transcriptional profiling of *PsHSP 18.1* and *PsHSP 71.2*. In addition, stipule samples were used for the quantification of ABA concentration and its five key ABA catabolites from the four major ABA catabolic pathways in ABA homeostasis by liquid chromatography-multiple reaction monitoring mass spectrometry. The expression of both HSP genes was up-regulated due to heat stress (HS) in anthers and stipules of all varieties, and the up-regulation was greatest at 3 h in stipules. In anthers, the induced transcription fold change of both genes was similar among different durations of HS. Likewise, more ABA accumulated in the ABA metabolism pool due to HS and the ABA response started rapidly after 3 hours of treatment. Heat tolerant varieties had a higher ABA synthesis and turnover rate at 3 h HS, than their respective heat susceptible counterparts. This study aids in understanding the impact of a warming summer on the pea crop in the perspective of HSP and ABA hormone regulation and gives a new insight to perceive the different heat tolerance among Canadian pea varieties.

3.2 Introduction

Pea (*Pisum sativum*) belongs to the cool-season legume family, among which pea is considered as the most heat susceptible (Siddique, 1999). In Canada, which accounts for one third of global pea production, lower grain yield was observed in summers when the daily maximum temperature exceeded 28°C during flowering, or the seasonal temperature was over 17.5°C (Bueckert et al., 2015). In plant cellular defense against HS, the induction of HSPs is one of the major responses. HSPs act as molecular chaperones to facilitate folding of other functional proteins and prevent them from denaturation and aggregation during exposure to HS. Depending on protein molecular weight, HSPs have been classified into HSP 100, HSP 90, HSP 70, HSP 60, and sHSP. In pea, HSPs have only been characterized in the subfamilies of sHSP and HSP70 (DeRocher et al., 1991; DeRocher & Vierling, 1995; Lenne et al., 1995). Among these reported pea HSP genes, the expression of *PsHSP 17.9*, *PsHSP 18.1*, *PsHSP 71.2* and *PsHSP 70b* transcripts appeared to be heat inducible (DeRocher et al., 1991; DeRocher & Vierling, 1995). Srikanthbabu et al. (2002) further provided evidence that the induction of *PsHSP 18.1* and *PsHSP 70* at moderately high temperature improved survival rate of pea seedlings at subsequent lethal HS.

The phytohormone ABA also plays an important role in mediating plant adaptation to stress. Temperature stress can activate numerous genes involved in ABA biosynthesis, catabolism and transport; ABA biosynthesis genes in Arabidopsis e.g., *ABA1*, *ABA2*, *ABA4*, *AAO3*, *NCED3* were differentially expressed (up- or down-regulated) in an organ-specific manner among stem leaves on the flowering nodes, inflorescence meristem, and developing silique. As a result, a significant accumulation of ABA was detected in 37°C treated leaves compared to leaves at 22°C (Baron et al., 2012). The induction of ABA under high temperature was observed after 8 h at 45°C in *Brassica napus* seedlings (Kurepin et al., 2008). ABA profiling in cold-stressed rice and drought-stressed wheat suggested that increased ABA accumulation within reproductive structures was negatively correlated with abiotic stress tolerance (Oliver et al., 2007; Ji et al., 2011). Little is known as to whether ABA metabolism or signalling responds differently between varieties contrasting in their heat tolerance. In general, plants have four ABA catabolic pathways, among which conjugation and hydroxylation of the 8'-carbon atom are the major pathways. The balance between ABA biosynthesis, catabolism and transport determines

ABA homeostasis in plant tissue. An ultra-high performance liquid chromatography-selected reaction monitoring mass spectrometry (UHPLC-SRM MS) approach was developed for the quantification of ABA and its major metabolites. In this study we are not only curious whether ABA itself accumulates in HS, but more importantly, how the ABA homeostasis would change with HS.

It is hypothesized that 1) both pea HSPs (*PsHSP 18.1 and PsHSP 71.2)* and stipule ABA are involved with pea response to HS, and 2) response variation would be observed among different pea varieties and the differentials would link with varieties' heat tolerance characterized at the field trial level.

3.3 Materials and methods

3.3.1 Plant material

Two pairs of heat tolerant and susceptible pea varieties, CDC Meadow vs. Nitouche and PR11-2 vs. PR11-90, had been identified from different genetic backgrounds in previous studies in Canada. CDC Meadow (Warkentin et al., 2007) demonstrated superior heat tolerance out of the 24 genotypes studied by Tafesse et al. (2019) and Jiang et al. (2019), as it had consistently superior performance in canopy temperature depression, high *in vitro* pollen germination under HS, and high grain yield in field trials. Nitouche, originating from DLF Trifolium in Denmark, is considered as heat sensitive due to its high ovule abortion rate and relatively poor yield performance under high temperature (Jiang et al., 2019). PR11-2 and PR11-90 are recombinant inbred lines at F8 from the population PR11, which was derived from the cross CDC Centennial (Warkentin et al., 2007) // CDC Sage (Warkentin et al., 2006) made in 2008 at the Crop Development Centre (CDC), University of Saskatchewan (Huang et al., 2017). Both varieties have white flowers and green cotyledons, but PR11-2 has higher pod number per plant, longer flowering duration and greater grain yield than PR11-90 based on field trials under normal temperature and heat stress conditions, and thus PR11-2 is considered as more heat tolerant than PR11-90 (Table 3.1).

Table 3.1. Characteristics of flowering and yield related traits of CDC Meadow, Nitouche, PR11-2 and PR11-90 in 2017-2019 (rep = 3 in each year trial) at Saskatoon, Canada.

variety	seeding date	DTF	DOF	RNN	PN	SNPP	TSW	plot yield(kg/ha)
CDC Meadow	normal	54.3	16.3	5.6	9.0	4.7	217	2784
	late	49.4	14.6	5.1	8.9	4.1	189	2762
Nitouche	normal	58.0	13.7	4.8	7.2	4.0	278	2534
	late	50.7	12.3	4.4	6.7	3.5	213	1816
PR11-2	normal	56.9	15.6	5.4	7.7	4.1	215	2828
	late	51.9	12.7	4.8	7.4	3.5	217	2665
PR11-90	normal	48.0	17.7	4.5	7.1	5.8	201	2289
	late	47.7	13.0	3.3	5.2	4.8	178	1145

Note: DTF, days to flowering; DOF, duration of flowering; RNN, number of reproductive nodes on main-stem; PN, pod number on main-stem; SNPP, seed number per pod; TSW, thousand seed weight (g). Late seeding trial is more heat stressful trial compared to normal seeding.

3.3.2 Experimental design and heat treatment

Seed samples of heat tolerant varieties CDC Meadow and PR11-2, and heat susceptible varieties Nitouche and PR11-90 were obtained from CDC, University of Saskatchewan. Three seeds of each variety were planted in a 3.8 L pot containing Sunshine mix #4 (Sun Gro, Seba Beach, AB, Canada) at control temperature condition, i.e., 22/16°C 16/8 h day/night. The three plants in one pot were bulked later as one biological replicate. A randomized complete block design with three biological replications of each variety was utilized. Starting from one week after seedlings were germinated, the plants were watered twice or three times per week based on the growth stage and water use of each variety. Pots were fertilized weekly with 100 ml of quick release fertilizer (20 N:20 P_2O_5:20 K_2O) prepared at a concentration of 3 g L^{-1}. At the stage when plants developed flower buds but prior to anther dehiscence, plants assigned to heat treatment groups were transferred from the control temperature chamber to a chamber at 38/16°C 16/8 h day/night for 3 h, 6 h, 12 h, or 24 h respectively. Then all the anthers and stipules on the first flowering node from the three plants within one pot were freshly frozen in liquid nitrogen and stored at -80°C freezer. The whole experiment yielded a respective library of 60 samples of each plant organ type from four varieties, five heat treatments and three biological replicates.

3.3.3 RNA extraction and qRT-PCR

For each organ sample, the extraction of total RNA was conducted using QIAGEN RNeasy plant mini kit. The quantity of extracted RNA was then determined by optical density at 260 nm and the OD260/OD280 absorption ratio using NanoDrop 8000 UV spectrophotometer. The remaining RNA of each tissue sample was then stocked at -80°C. Total RNA (1 µg) was reverse transcribed to cDNA using SensiFAST cDNA synthesis kit (Bioline, Inc.). Four reference genes (*PP2A, GH720808, β-tubulin and GH720838*) were recommended for abiotic stress experiments in pea (Die et al., 2010). *GH720808,* encoding histone H3, and *GH720838,* encoding transcription factor IIA, were tested in this experiment, and *GH720838* was selected as the reference gene to normalize the relative expression quantities of the target genes, because it had the consistent expression among different time points and genotypes, i.e., among anther samples, cycle threshold (Ct) value was 19.44 ± 0.63, cv = 3.3; among leaf samples, Ct value was 20.65 ± 0.66, c v= 3.2. Specific primers of *PsHSP 18.1 and PsHSP 71.2* were designed via IDT Primer quest tool (Integrated DNA Technologies, Inc) with the following criteria: Tm of 62 ± 1°C, PCR amplicon lengths of 90-120 bp, primer length of 20-22 bp and GC contents of 45–55%. Primer efficiency (%) of each gene was equaled to $(10^{-1/slope} - 1) * 100$, and all primers had their efficiency rates between 90–110% and qualified for assay use (Appendix A). Then SensiFAST SYBR No-ROX kit was used for the target gene expression using an optical 384 well plate on BIO-RAD CFX384 real-time PCR machine in accordance with the manufacture's protocols.

3.3.4 Extraction of ABA and its major catabolites and quantification by ultra-high performance liquid chromatography-selected reaction monitoring mass spectrometry

In parallel, additional stipule samples were freeze-dried and ground into dry fine powder. Sixty stipule samples were prepared by weighing ~50 mg into a 2-mL microtube for the quantification of ABA and its catabolites, using UHPLC-SRM MS. For a negative control, a non-heat stressed stipule sample of the pea 'wilty' mutant line JI 1069 was used. JI 1069 was documented as an ABA deficient variety at drought stress (Wang et al., 1984).

One mL of solvent consisting of 80:19:1 methanol: water: formic acid containing deuterated internal standards (d_6–ABA, d_3–PA, d_3–DPA, d_4–7-OH-ABA, and d_5–ABA GE; Toronto Research Chemicals, Toronto) was added to each tube. After vortexing the tube for 5-10

s, the samples were placed onto a Thermo mixer for 30 min at 1400 rpm (room temperature). Samples were then centrifuged for 5 min at 12,000 rpm. An 800 µL aliquot of the supernatant was transferred into a new 2-mL microtube. A second extraction was carried out by adding 500 µL of the extraction solvent with no internal standard. After vortexing, mixing and centrifuging as described above, 500 µL from the second extraction was transferred and combined with the first extraction. 390 µL of the supernatant was transferred to a new micro tube and dried using a speed vac (Labconco Corp., Kansas City, MO). The dried sample was then reconstituted in 130 µL of 79:20:1 water: methanol: formic acid, vortexed and then mixed on the Thermo mixer at room temperature for 30 min at 1400 rpm. A volume of 110 µL was transferred to a HPLC vial containing a 150 µL insert prior to UHPLC-SRM MS.

ABA and its five metabolites, i.e., phaseic acid (PA) and dihydrophaseic acid (DPA) via C'8 hydroxylation, neophaseic acid (neoPA) via C'9 hydroxylation, 7'-OH ABA via C'7 hydroxylation and abscisic acid-glucose ester (ABA-GE) via β-D-glucopyranosyl conjugation, were selected for quantification. Five deuterated standards were selected as internal standards, and an eight-point calibration curve was prepared for quantification (Table 3.2).

Table 3.2. Selected reaction monitoring parameters for ABA and its selected catabolites on a Thermo Fisher Altis triple-quadrupole mass spectrometer.

Compound	Retention time (min)	Precursor→fragment (m/z)	CE (V)	RF Lens (V)
ABA	7.3	263 → 153	10.2	48
PA	4.8	279 → 139	12	54
DPA	2.4	281 → 171	18	73
7'-OH-ABA	5.8	279 → 151	15.2	47
ABA-GE	4.6	425 → 263	10.2	77
neo-PA	6.4	279 → 205	12	56
d_6-ABA	7.3	269 → 159	10.2	48
d_3-PA	4.7	282 → 142	10.2	53
d_5-ABA-GE	4.5	430 → 268	10.2	65
d_4-7'-OH-ABA	5.8	283 → 221	10.2	48
d_3-DPA	2.4	284 → 240	13	69

UHPLC-SRM MS was performed on a TSQ Altis triple quadrupole mass spectrometer equipped with a Vanquish Flex UHPLC (Thermo Fisher Scientific, San Jose, CA) in the negative

ion mode. A volume of 10 µL was injected from the vial and analytes were separated using an Agilent Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 µm) and 5 mm guard column. The mobile phase consisted of 1% formic acid in water (solvent A) or in 90% acetonitrile (solvent B). The flow rate was set to 0.4 mL/min and a 17-min solvent gradient was used. The gradient started with 10% solvent B and gradually increased to 20% at 5 min, and to 30% at 7.0 min, it then was increased to 42% at 11 min, ramped to 90% at 12 min and then maintained for 2 mins. It was returned to 10% at 14.5 min and maintained for 2.5 min to equilibrate the column. The LC column temperature was controlled at 25°C and the autosampler at 6°C. The mass spectrometer parameters were: electrospray voltage 2500 V, vaporizer temperature 350°C, sheath gas pressure 60, auxiliary gas pressure 10, sweep gas 1, and capillary temperature 325°C. The SRM parameters, such as the selection of precursor and fragment ions, collision energies and RF lens for the compounds are shown in Table 3.2.

3.3.5 Data quality check and outlier data removal

For the dataset of each measured parameter from individual heat treatments, outliers were filtered among varieties according to Tukey's criteria. An outlier was defined as any data outside the range [Q1-1.5*(Q3-Q1), Q3+1.5*(Q3-Q1)], where Q1 and Q3 are the lower and upper quartiles, respectively.

3.3.6 qRT-PCR data analysis

For each time point, transcription fold change (fc) of HSP gene relative to reference gene is demonstrated as $2^{(-\Delta Ct)}$, where ΔCt = (Ct of HSP gene – Ct of reference gene) (Schmittgen and Livak, 2008). Mean transcriptional fc of *PsHSP 18.1* gene and *PsHSP 71.2* among different time points were compared using SAS Proc Mixed model (Version 9.4, SAS Institute Inc. Cary, NC, USA). Following that, fold change values were further normalized via $2^{-\Delta\Delta Ct}$ method in order to compare the two genes' transcription changes among different HS periods between stipules and anthers among four genotypes.

3.3.7 Statistical analysis

For the concentration of ABA and its five catabolites, individual analysis of variance was conducted via proc mixed model in SAS 9.4 (SAS Institute Inc. Cary, NC, USA). Multiple variety means were compared based on least significant difference at significance level 0.05.

3.4 Results

3.4.1 Transcriptional profiling of *PsHSP 18.1* and *PsHSP 71.2* genes between control temperature and heat stress

In the control temperature environment, the transcriptional levels of both *PsHSP 18.1* and *PsHSP 71*.2 were minimal compared to the transcription of the reference gene *GH720838* on both anther and stipule samples (Table 3.3). Whereas their transcription levels were significantly induced due to HS (Fig 3.1). Our results supported previous classification of both genes as heat inducible. The transcriptional level of *PsHSP 18.1* was greater than that of *PsHSP 71*.2 in both stipules and anthers at control temperature.

Table 3.3. Relative *PsHSP 18.1* and *PsHSP 71*.2 gene expression to the reference gene *GH720838* on non-heat stressed anther and stipule samples.

variety	stipules		anthers	
	PsHSP 18.1	*PsHSP 71.2*	*PsHSP 18.1*	*PsHSP 71.2*
PR11-2	0.14	0.08	0.33	0.12
PR11-90	0.07	0.05	0.24	0.05
CDC Meadow	0.17	0.05	0.24	0.06
Nitouche	0.17	0.11	0.35	0.10
Mean	0.14±0.05	0.07±0.03	0.29±0.06	0.08±0.03

Note: expression fold change of HSP gene relative to reference gene is demonstrated as $2^{(-\Delta Ct)}$, where ΔCt = (Ct of HSP gene – Ct of reference gene); each mean fold change shown in the last row is the average across the four varieties.

When subjected to HS, the transcription of both genes was significantly increased in all anther and stipule samples of all four varieties at every time point measured. In stipules, both genes had greater expression at 3 h compared to 6 h, 12 h and 24 h (Fig 3.1c, 3.1d). At each time point under HS, the induction fold change of both genes was statistically similar among the four varieties. In anther samples of each variety, the induction fold change of *PsHSP 71.2* was similar among the four time points measured (Fig 3.1a). PR11-90 and Nitouche had greater induction than PR11-2 and CDC Meadow at some time points, e.g., 3 h at 38°C. For the heat response of *PsHSP 18.1* in anthers, the transcriptional induction was at similar fold change across the four time points in both PR11-2 or Nitouche (Fig 3.1b). Whereas in CDC Meadow, *PsHSP 18.1* had the lower fold change at 6 h but similar fold changes at 3 h, 12 h and 24 h. In the anthers of

PR11-90, *PsHSP 18.1* had less induction at 24 h compared with the induction levels at 3 h and 12 h. For individual time points under HS, the transcriptional fold change of *PsHSP 18.1* had no significant variation at 24 h among four varieties. At 3 h, the transcription of *PsHSP 18.1* was more induced in PR11-90 than PR11-2. At 6 h, PR11-90 had greater expression of *PsHSP 18.1* than PR11-2 and CDC Meadow. At 12 h, CDC Meadow and PR11-90 had greater induction than Nitouche and PR11-2.

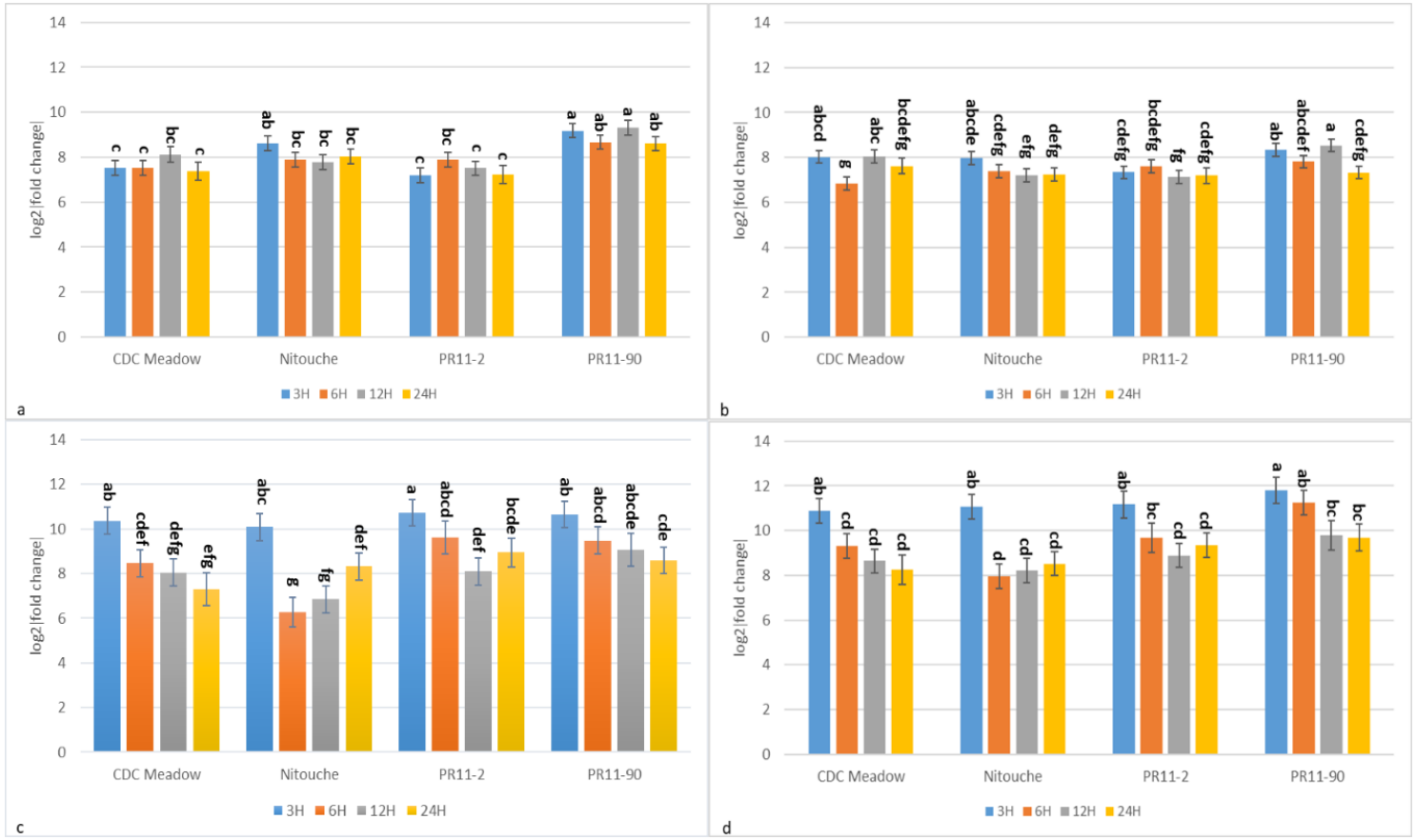


Fig 3.1. Transcriptional response of *PsHSP 71.2* and *PsHSP 18.1* at 3 h, 6 h, 12 h or 24 h 38°C heat stress in whole anthers (panel a for *PsHSP 7*1.2, b for *PsHSP 18.1*) and stipules (panel c for *PsHSP 71.2*, d for *PsHSP 18.1*) at the same flowering node among four pea varieties. Each bar represents average gene transcriptional fold change in log2 ($2^{-\Delta\Delta Ct}$) across three biological replications and two technical replications, and error bar represents standard error. Within a panel, mean values followed by the same letter did not differ significantly at $P < 0.05$.

3.4.2 Genetic variation in stipule ABA homeostasis under control temperature condition

Total concentration, that is the sum of ABA and its catabolites, was documented as an amount accounting for the ABA pool. The total ABA concentration of the negative control, JI 1069, was profiled to be 1.1 nmol/g dry weight in our assay, which was much lower than the total ABA concentration of the four varieties of interest at control temperature (mean = 11.4 nmol/g dry). A means comparison of individual ABA catabolites and the total concentration among the four varieties was conducted on non-heat stressed stipule samples. Even at the control temperature condition, significant variety differences were detected in DPA, neoPA and the total ABA pool concentration (Table 3.4). However, these variations among the four varieties did not depend on their heat tolerance classification. One heat susceptible variety, Nitouche, had the highest concentration of all ABA catabolites and the total ABA pool, but the other susceptible variety PR11-90 had the second lowest DPA and total concentrations. The heat tolerant cultivar CDC Meadow had the lowest concentrations of DPA and the total ABA pool among the four varieties, but PR11-2 had relatively high concentrations.

Table 3.4. Means ± standard deviations of ABA, ABA catabolites and total ABA pool concentration on non-heat-stressed stipule samples among four pea varieties.

variety	heat tolerance	ABA	DPA	PA	7'-OH ABA	ABA-GE	neoPA	total
CDC Meadow	tolerant	1.20±0.45a	5.99±1.08b	0.72±0.10a	0.32±0.18a	0.02±0.00a	0.06±0.01b	8.30±1.39c
Nitouche	susceptible	1.49±0.40a	11.02±1.05a	0.63±0.02a	0.18±0.06a	0.02±0.00a	0.09±0.01a	13.40±0.79a
PR11-2	tolerant	1.41±0.32a	10.47±2.37a	0.86±0.04a	0.23±0.15a	0.03±0.01a	0.05±0.01b	13.05±1.95ab
PR11-90	susceptible	1.05±0.40a	8.66±0.37b	0.70±0.20a	0.22±0.05a	0.03±0.02a	0.04±0.02a	10.70±0.60bc

Note: Values are in units of nmol/g dry weight; for each column, if the values share a common letter, it means that they are not significantly different at 0.05.

3.4.3 ABA homeostasis response towards high temperature

To understand the whole ABA pool response to HS, ANOVA on the effects of varieties and different duration of HS on the total amount of ABA metabolites showed that varieties and high temperature treatment both had significant effects (Table 3.5). More ABA in the pool accumulated at all heat treatments compared to the control temperature, with double peaks at 3 h and 24 h (Table 3.6). Although the whole ABA pool concentration at 3 h and 24 h 38°C were similar, the composition of the ABA pool was different. At 3 h 38°C, DPA accounted for 85% of the total; at 24 h 38°C, DPA only accounted for around 50% of the total whereas a large amount of ABA was synthesized, implying a new round of ABA signaling is about to conduct.

Regarding ABA and its various metabolites, ANOVA results showed that ABA, DPA, PA, ABA-GE and neoPA had significant responses to temperature treatment, whereas 7'-OH ABA had no response (Table 3.5). When pea stipules were exposed to 3 h HS, ABA concentration dropped significantly compared to control temperature, and it was converted to DPA via the 8'-hydroxylation pathway; as a result, DPA concentration increased accordingly (Table 3.6). At 6 h and 12 h, ABA concentration increased to its similar level of control temperature. For neoPA derived from ABA via 9'- hydroxylation, its concentration did not vary between control temperature and HS at 3 h, 6 h and 24 h, but its concentration decreased at 12 h 38°C.

Table 3.5. Analysis of variance with F values on the effect of varieties, different hours of heat stress and their interactions on the amount of ABA, ABA catabolites and the total concentration in pea stipules of four varieties.

Factor	Num DF	F values						
		ABA	DPA	PA	7'-OH ABA	ABA-GE	neoPA	total
variety	3	0.74ns	12.58***	11.58***	7.77***	2.06ns	1.88ns	11.49***
treatment	4	16.33***	9.58***	86.17***	1.74ns	16.17***	4.39**	5.88***
var*trt	12	0.35ns	0.54ns	6.05***	0.76ns	2.04*	2.48*	0.58ns

Note: ns, not significant; *, significant at 0.05; **, significant at 0.01; ***, significant at 0.001.

Table 3.6. Average concentrations among four varieties of ABA, ABA catabolites and total ABA pool in stipules at control temperature 22°C and 3 h, 6 h, 12 h and 24 h at 38°C.

Treatment	ABA	DPA	PA	7'-OH ABA	ABA-GE	neoPA	total
0h	1.29±0.50b	9.03±2.38bc	0.73±0.14d	0.24±0.12a	0.03±0.01b	0.06±0.02a	11.37±2.42b
3h	0.43±0.13c	12.64±3.05a	1.42±0.25c	0.35±0.17a	0.05±0.02b	0.07±0.02a	14.95±3.05a
6h	1.09±0.61b	8.93±2.83bc	1.80±0.94b	0.25±0.11a	0.04±0.01b	0.07±0.04a	12.19±3.92b
12h	1.43±0.73b	9.71±1.92b	1.03±0.42d	0.28±0.10a	0.08±0.04b	0.04±0.01b	12.56±2.60b
24h	3.80±1.98a	7.91±2.07c	3.32±0.88a	0.32±0.10a	0.31±0.25a	0.06±0.02a	15.71±3.95a

Note: Values are average concentrations among four varieties in the unit of nmol/g dry weight; for each column, if the values in each column share a common letter, it means that they are not significantly different at 0.05.

3.4.4 ABA response variation between heat tolerant and heat susceptible pairs of varieties

The concentration changes of ABA, its catabolites and total ABA pool between control temperature and different heat treatments was characterized for each of the four varieties. For each variety, the ABA concentration was the lowest after 3 h 38°C (Fig 3.2). The ABA pool concentration was the highest at 3 h and 24 h at 38°C, which was consistent with the result in Table 3.6. A contrasting pattern of stipule total ABA pool concentration between the two pairs of heat tolerant and heat sensitive varieties was observed. In the pair of CDC Meadow and Nitouche, CDC Meadow, the more heat tolerant one, had a lower level than Nitouche (susceptible counterpart); whereas in the pair of PR11-2 and PR11-90, heat tolerant PR11-2 had a higher level than PR11-90. But from the perspective of ABA response to heat treatment, at 3 h HS, the total ABA pool concentration of CDC Meadow increased by 53% compared to control temperature, and the corresponding increase percentage was 12%, 36% and 33% for Nitouche, PR11-2 and PR11-90, respectively. The increased percentage of ABA converting to DPA was 74%, 18%, 48% and 35% for CDC Meadow, Nitouche, PR11-2 and PR11-90, respectively. Both PR11-2 and CDC Meadow had a faster ABA turnover rate, with more ABA synthesised in the pool after 3 hours at 38°C than their sensitive counterparts. The similar response pattern in the two groups suggests that heat tolerance might correlate with a greater ABA induction and a faster turnover rate at the early stage of HS.

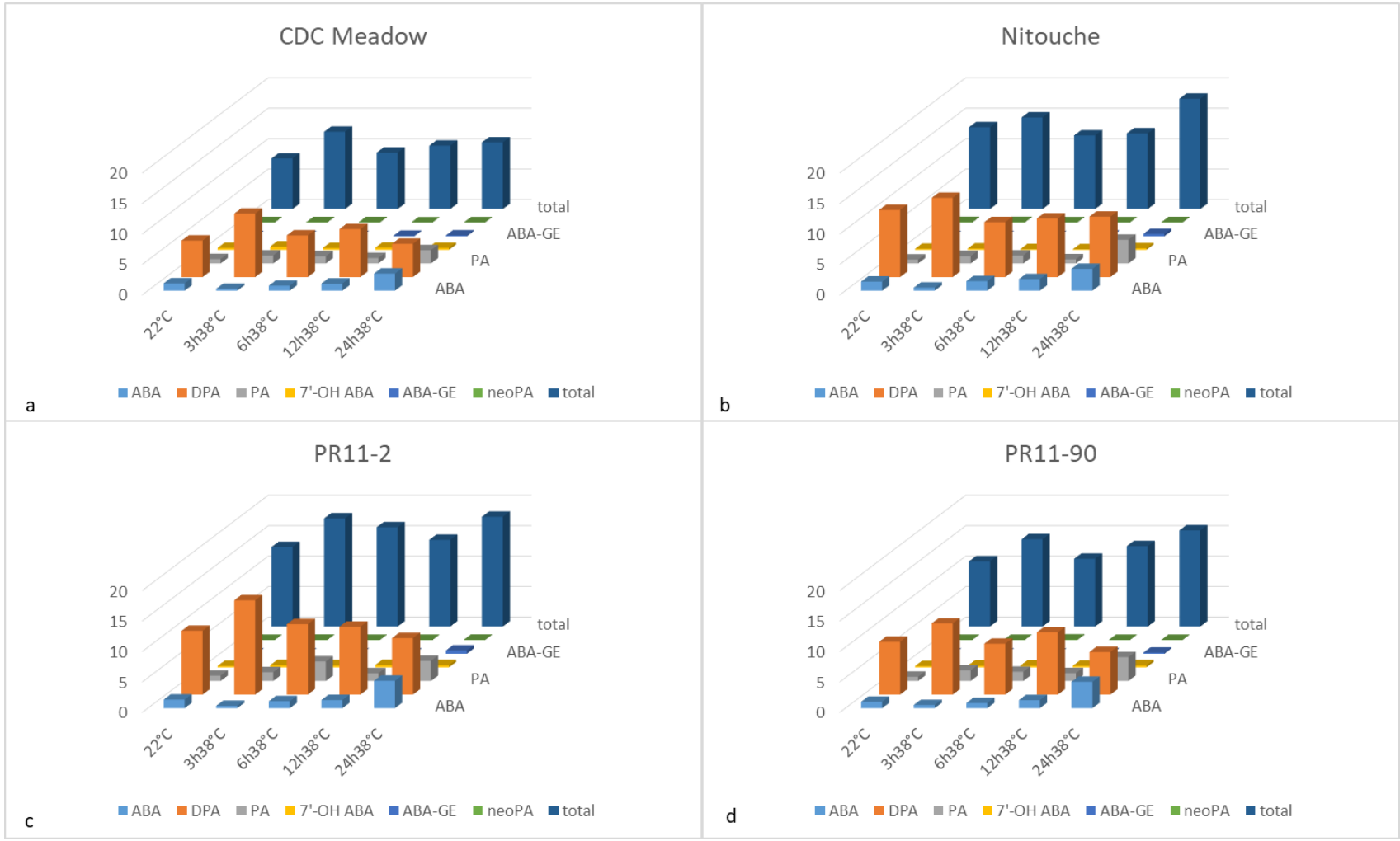


Fig 3.2. The concentration (nmol/g dry weight) change of ABA, PA, DPA, 7'-OH ABA, ABA-GE, neoPA and the total over different duration of heat stress at 38°C on stipules of two heat tolerant pea varieties, CDC Meadow (panel a) and PR11-2 (panel c), and two heat susceptible pea varieties, Nitouche (panel b) and PR11-90 (panel d).

3.5 Discussion

3.5.1 Induced transcription of *PsHSP 18.1* and *PsHSP 71.2* at heat stress

Based on their expression patterns, HSPs within each family can be classified into three sub-categories, which are heat-induced, expressed constitutively but not heat-induced, and expressed constitutively with additional heat induction. The two HSP genes, *PsHSP 18.1* and *PsHSP 71.2*, whose expression was identified as heat inducible on a vegetable pea variety (DeRocher et al., 1991; DeRocher and Vierling, 1995), were validated in this study on field pea varieties with various thermal tolerance. Both genes were minimally expressed in the stipules and anthers at non-stressed control temperature condition (Table 3.3), but their expression was dramatically induced when subjected to high temperature treatment (Fig 3.1).

In plant cellular defense against heat, the induction of HSP is one of the major responses. HSPs act as molecular chaperones which are proteins that facilitate folding of other functional proteins especially at the secondary and tertiary structure during non-stress periods and prevent them from denaturation and aggregation during exposure to HS. Overexpression of HSP genes in transgenic plants helped plants exhibit improved tolerance to high temperature (Fragkostefanakis et al., 2015; Mishra et al., 2018). For stipule samples in this study, 3 h high temperature treatment was the most heat responsive time point as the fold change of both genes reached the maximum on every variety (Fig 3.1c, 3.1d). This rapid response corresponded with the characterization of *PsHSP 18.1* and *PsHSP 71.2* in the vegetable pea variety, Little Marvel (DeRocher et al., 1991; DeRocher and Vierling, 1995) as well as the findings reported in other plant species (Wahid et al., 2007). For anthers of individual varieties, the transcriptional level of both genes was steady during HS (Fig 3.1a, 3.1b). Greater expression was seen in stipule than anther, suggesting a better thermal tolerance in vegetative organs than in reproductive organs. However, the induction threshold of both genes did not correlate with the varieties' heat tolerance classification. In heat stressed stipules, CDC Meadow and PR11-2 had similar accumulation levels of both transcripts as their susceptible counterpart, Nitouche and PR11-90, at individual time points. And in anthers, both genes had higher induction in PR11-90 than PR11-2 at many time points, *PsHSP 71.2* in particular. Heat induced transcription of HSP genes could be cultivar specific in rice (Chandel et al., 2013) and bread wheat (Mishra et al., 2017), when screening among multiple heat tolerant and susceptible varieties. Similar to this study, the

differential induction of HSP genes did not depend on the classification of the variety's heat tolerance. They both found heat tolerant variety and susceptible variety could have strong induction in some of HSP genes. One possible explanation on high HSP induction in a heat susceptible variety is that a rescue mechanism is taking place.

3.5.2 Pea ABA metabolic homeostasis at control temperature and heat stress

In the limited papers related to pea ABA, leaf ABA concentrations were reported in the range of 0.76-2.27 nmol/g dry weight (Zhang & Davies, 1987; Zhang & Zhang, 1994), and the ABA concentrations in this experiment fell within the range (Table 3.4). DPA derived from C-8' hydroxylation was the most abundant among ABA catabolites, whereas neoPA via 9' hydroxylation and 7'OH-ABA via 7' hydroxylation were minimal. Though there was no resource in pea to compare, in a general review of plant papers, 8' hydroxylation is believed to be the dominant catalytic hydroxylation pathway (Zeevaart and Creelman, 1988; Cutler and Krochko, 1999; Sah et al., 2016). At control temperature, free ABA levels in stipules were not statistically different among four pea varieties, which was in agreement with the finding of Ji et al. (2011) that there was no significant variation of endogenous ABA levels in unstressed leaves among four wheat varieties with varying drought tolerance. However, in our study, significant variety differences were detected in DPA, neoPA and the total ABA pool concentrations (Table 3.4).

ABA mediated thermotolerance was considered independent of HSP heat response. Seven-day old seedlings of Arabidopsis *aba1*, *aba2* and *aba3* mutants with deficiency in different ABA synthesis genes displayed reduced basal and acquired heat tolerance compared with wild type (Larkindale et al., 2005). In our study, JI1069, an ABA-deficient variety, demonstrated a wilting phenotype under control temperature, particularly during reproductive stage, and as a result fruit production failed. Its heat susceptibility compared with other pea varieties was mainly due to low vigour at control temperature. When subjected to HS, ABA response could be rapid, i.e., within hours. Leaf ABA concentration started to increase within the first hour of HS in grape leaf (Abass and Rajashekar, 1993), and during the 4 h-8 h period of HS in canola leaf (Kurepin et al., 2008). Although we found ABA pool concentration accumulated at 3 h of high temperature, the induction was not continuous (Table 3.6). Its concentration between 6 h and 12 h HS dropped to the similar level of control temperature, but the concentration started

to increase again during 12-24 h HS. ABA pool accumulation after 24 h HS was mainly due to the significant increase in the concentration of active ABA. This induced active ABA was also found in *A. thaliana* leaves when 24 h HS was applied (Baron et al., 2012). Active ABA concentration at 3 h HS, on the contrary, was repressed among four pea varieties. The accumulated ABA pool concentration at this time was due to increased DPA via ABA C'8-hydroxylation pathway. Interestingly, heat tolerant varieties, CDC Meadow and PR11-2, had faster ABA turnover than their susceptible counterpart varieties, which appears to be linked with pea heat tolerance. Similarly, a faster ABA turnover was reported in a heat tolerant rice variety (Tang et al., 2008), a cold-tolerant rice variety (Oliver et al., 2007) and two drought tolerant wheat varieties (Ji et al., 2011) compared to their susceptible checks.

3.6 Conclusions

The thesis general hypothesis I 'HSP genes and ABA homeostasis would respond rapidly in HS and the response would be different between pea heat tolerant and heat susceptible varieties', was partially accepted from the results of this chapter. The transcription of *PsHSP 18.1* and *PsHSP 71.2* was most induced at 3 h at 38°C in stipules. In anthers, the induction of both genes was similar at 3 to 24 hours of HS. Compared to control temperature, the average ABA pool concentration among four varieties increased by more than 30% at 3 h and 24 h of HS. Heat tolerant varieties had greater ABA response than heat susceptible varieties in terms of 25% faster ABA turnover rate at early HS stage. This response differential between tolerant and susceptible varieties linked well with different heat tolerance of the four varieties at the field level. However, HSPs response appeared to be a conserved plant HS response as the expression induction did not differ significantly among varieties known to differ in heat tolerance under field conditions.

Transition section between Chapter 3 and Chapter 4

Heat response of two pea HSP genes and ABA homeostasis was characterized among four pea varieties. In general, both HSP genes and ABA homeostasis had highest responses after 3 h at 38°C among the four pea varieties in this study, thus the 3 hours heat treatment was selected for the subsequent transcriptional profiling experiment in chapter 4. The objective of transcriptional profiling via RNA-Seq is to characterize the full set of heat responsive genes in pea. Because the transcription differential of *PsHSP 18.1* and *PsHSP 71.2* between PR11-2 and PR11-90 was greater than in the pair of CDC Meadow and Nitouche, PR11-2 and PR11-90 were chosen as pea varieties in the RNA-Seq experiment.

CHAPTER 4. IDENTIFICATION OF HEAT RESPONSIVE GENES IN PEA STIPULES AND ANTHERS THROUGH TRANSCRIPTIONAL PROFILING

This chapter was published in PLOS One in November 2021.

Full citation: **Huang, S**., Gali, K.K., Lachagari, R.V.B., Chakravartty, N., Bueckert, R.A., Tar'an, B., and Warkentin, T.D. (2021) Identification of heat responsive genes in pea stipules and anthers through transcriptional profiling. PLoS ONE 16(11): e0251167; https://doi.org/10.1371/journal.pone.0251167.

4.1 Abstract

Field pea (*Pisum sativum* L.), a cool-season legume crop, is known for poor heat tolerance. Our previous work identified PR11-2 and PR11-90 as heat tolerant and susceptible lines in a recombinant inbred population. CDC Amarillo, a Canadian elite pea variety, was considered as another heat tolerant variety based on its similar field performance as PR11-2. This study aimed to characterize the differential transcription. Plants of these three varieties were stressed for 3 h at 38°C prior to self-pollination, and RNAs from heat stressed anthers and stipules on the same flowering node were extracted and sequenced via the Illumina NovaSeq platform for the characterization of heat responsive genes. *In silico* results were further validated by qPCR assay. Differentially expressed genes (DEGs) were identified at log2 |fold change (FC)| ≥ 2 between high temperature and control temperature. The three varieties shared 588 DEGs which were up-regulated and 220 genes which were down-regulated in anthers when subjected to heat treatment. In stipules, 879 DEGs (463/416 upregulation/downregulation) were consistent among varieties. The above heat-induced genes of the two plant organs were related to several biological processes i.e., response to heat, protein folding and DNA templated transcription. Ten gene ontology (GO) terms were over-represented in the consistently down-regulated DEGs of the two organs, and these terms were mainly related to cell wall macromolecule metabolism, lipid transport, lipid localization, and lipid metabolic process. The GO enrichment analysis on distinct

DEGs of individual pea varieties suggested that heat affected biological processes were dynamic, and variety distinct responses provide insight into molecular mechanisms of heat-tolerance response. Several biological processes, e.g., cellular response to DNA damage stimulus in stipule, electron transport chain in anther that were only observed in heat induced PR11-2 and CDC Amarillo, and their relevance to field pea heat tolerance, is worth further validation.

4.2 Introduction

Human activities have contributed approximately 1°C temperature increase globally since the Industrial Age and are predicted to cause another 0.5–1°C increase in the period between 2030 and 2052 according to current greenhouse gas emission rates (Hoegh-Guldberg et al., 2018). The evidence of the rising temperature causing lowered grain production was reported in the three major crops, maize, wheat, and rice (Lobell and Field, 2007). Heat stress (HS) also limits the production on legume crops including pea. In Canada, where its pea production accounts for one third of the global production, lowered grain yield was observed in summers when the maximum temperature exceeded 28°C during flowering, or the seasonal temperature was over 17.5°C (Bueckert et al., 2015; Huang et al., 2017). Because of the concern about a warming summer in North America, physiological studies on HS related damage on field pea, particularly the reproductive plant parts, have been conducted in the last decade. Under growth chamber conditions, when pea plants at anthesis were subject to seven-days HS of 36/18°C day/night, their pollen development, pollination and subsequent seed set were dramatically impaired compared to pea plants exposed to normal conditions of 24/18°C (Jiang et al., 2015). In addition, pea anther dysfunction was significantly induced after HS exposure (Jiang et al., 2019), and the impairment of anther development due to HS was similarly seen in cowpea (Ahmed et al., 1992) and common bean (Porch and Jahn, 2001). Pea leaf physiological functions could also be impaired by HS. The chlorophyll variable fluorescence, a measure of injury to photosynthesis, declined an average 8% after 3 days at 30/15°C in five pea cultivars, compared with the control temperature at 20/15°C in the pea cultivar Alaska (McDonald and Paulsen, 1997). In terms of pea breeding, progress has also been made in the characterization of heat tolerance based on field trials. A longer duration from sowing to flowering termination, and a greater pod production per plant contributed to increased grain yield potential at both hot and normal conditions, and several stable quantitative trait loci were characterized related to flowering and yield component traits (Huang et al., 2017). Lodging resistance and the semi-leafless leaf type resulted in a cooler pea

canopy and a greater yield potential (Tafesse et al., 2019). Additionally, the authors further characterized putative genomic loci of heat responsive traits, e.g., canopy temperature, pod number and chlorophyll concentration, via a pea genome wide association mapping study (Tafesse et al., 2020).

The discovery of heat responsive genes started with the characterization of heat shock protein (HSP) genes and heat shock factors (HSFs). Findings in this aspect were firstly well documented in *Arabidopsis thaliana*. In addition to the 21 known HSFs (Nover et al., 2001), the Arabidopsis heat stress response (HSR) is partly mediated by 13 HSP20s (Scharf et al., 2001), 18 HSP70s (Lin et al., 2001), seven HSP90s (Krishna and Gloor, 2001), and up to eight members of the HSP100s (Agarwal et al., 2001). The gene family of HSP20 was the most highly expressed under HS, followed by the gene family of HSP70 and HSP90, and the gene family of HSP100 was not responsive to HS (Swindell et al., 2007). Subsequent studies on the global transcriptome profiling under HS revealed that heat responsive genes could expand to those other genes involved in plant hormone biosynthesis and signaling, calcium and sugar signaling, primary and secondary metabolism (Endo et al., 2009; Frey et al., 2015; Yu et al., 2014). Cell wall and secondary metabolite pathways were also highly affected under HS in lentil (Singh et al., 2019). However, both the number of up- and down-regulated genes and the ratio of up- and down-regulated genes under HS varied among the aforementioned studies depending on HS treatments, plant species, genotypes and different plant organs used for RNA isolation.

The research on heat responsive gene discovery in pea is limited to the findings of HSP genes. Among the reported pea HSP genes, the expression of *PsHSP18.1* and *PsHSP71.2* genes appeared to be heat inducible (DeRocher and Vierling, 1995; DeRocher et al., 1991). The relation of HSPs to heat tolerance was subsequently confirmed as the induction of these HSP genes improved survival rate of pea seedlings and mature plants at high temperature (Srikanthbabu et al., 2002). Moreover, several HSP genes had a greater heat-induced expression in one of the heat tolerant cultivars, Acc.623, than in one susceptible variety Acc.476.

Particular attention to transcriptomic characterization under HS is required for a better understanding of pea HSR at the gene level. Although lacking the reference genome previously, transcriptome profiling via RNA-Seq studies were carried out in pea over the last decade, mainly focusing on the mining of genetic markers. The first pea transcriptome reference was developed

using next generation sequencing with the Roche/454 platform (Franssen et al., 2011). Later Illumina high-throughput sequencing was applied to sequence 23 cDNA libraries from multiple tissues of the Australian field pea cultivars Kaspa and Parafield (Sudheesh et al., 2015). A large proportion of the assembled contigs were expressed in both cultivars. To date, no transcriptome-wide mapping of pea response to HS has been conducted, but this method was utilized in the discovery of responsive genes in field pea seed aging (Chen et al., 2013), root nodulation (Alves-Carvalho et al., 2015) and most recently in water-logging stress studies (Henriet et al., 2019; Zaman et al., 2019). The utilization of RNA-Seq technique in pea HS research allows for the genome-wide mining of heat responsive genes and the global description of the complex regulatory pathway in the protection against HS at the cellular level, as well as comparative analysis of genes responsive to HS among different pea varieties, or between pea and other crop species. Thus, the objectives of this research included 1) the characterization of additional gene responses toward high temperature besides previously characterized pea HSP genes; 2) the comparative analysis of heat responsive gene expression differences between anthers, representative of the reproductive plant parts, and stipules, representative of the vegetative plant parts; 3) the comparison between heat tolerant and heat susceptible varieties for an enhanced understanding of pea heat tolerance and susceptibility from the view of gene response. It is hypothesized that the application of the RNA-Seq method would fulfill the aforementioned purposes.

4.3 Materials and methods

4.3.1 Plant materials

Three pea varieties were used as plant material for this experiment, that is, PR11-2 (heat tolerant variety), PR11-90 (heat susceptible variety) and CDC Amarillo (check variety). PR11-2 and PR11-90 are recombinant inbred lines from the population PR11, which was derived from the cross CDC Centennial/CDC Sage made in 2008 at the Crop Development Centre (CDC), University of Saskatchewan (Huang et al., 2017). CDC Centennial was developed at CDC. It is a high yielding yellow pea cultivar with a semi-leafless leaf type with moderately large seeds (Warkentin et al., 2007). CDC Sage is a high yielding cultivar from the CDC with green cotyledons and medium-small seeds (Warkentin et al., 2006). PR11-2 and PR11-90 have white flowers and green cotyledons, but PR11-2 has a greater pod number per plant, longer flowering

duration and greater grain yield than PR11-90 based on field trials at both normal and hot conditions (T-test at 0.05 significance level), thus PR11-2 is considered to have better heat tolerance than PR11-90 (Table 4.1). CDC Amarillo (Warkentin et al., 2014), a yellow pea variety and one of the best yielding varieties in western Canada, was included as a check. Because CDC Amarillo has a similar field performance as PR11-2 in our field test at normal and heat stressful conditions (Table 4.1), it is also considered as heat tolerant compared with PR11-90.

Table 4.1. Characteristics of flowering and yield-related traits of PR11-2, PR11-90 and CDC Amarillo at normal and late seeding trials in 2017-2019 at Saskatoon, Canada.

Variety	seeding date	DTF	DOF	RNN	PN	SNPP	TSW(g)	plot yield(kg/ha)
PR11-2	normal	56.9	15.6	5.4	7.7	4.1	215.4	2827.7
	late	51.9	12.7	4.8	7.4	3.5	216.6	2665.2
PR11-90	normal	48.0	17.7	4.5	7.1	5.8	200.9	2288.9
	late	47.7	13.0	3.3	5.2	4.8	177.6	1144.9
CDC Amarillo	normal	56.5	14.5	5.0	7.6	4.6	236.4	3064.5
	late	51.4	13.6	4.4	7.1	3.9	200.9	2734.2

Note: DTF, days to flowering; DOF, duration of flowering; RNN, reproductive node number on main-stem; PN, pod number on main-stem; SNPP, seed number per pod; TSW, thousand seed weight (g). Late seeding trial is more heat stressful trial.

4.3.2 Experimental design

A randomized complete block design experiment utilizing the three varieties with three biological replicates and two temperature treatments was carried out in a phytotron chamber in the Agriculture Building, University of Saskatchewan. Temperature treatments consisted of a control temperature treatment 24/18°C, 16/8 h day/night (Jiang et al., 2015), and a high temperature treatment 38/18°C, 16/8 h day/night (DeRocher et al., 1991). The control temperature regime represents typical non-stress western Canadian pea field conditions in summer. Three seeds of each variety were planted in individual 3.8 L pots containing Sunshine mix #4 (Sun Gro, Seba Beach, AB, Canada). The three plants in one pot were bulked later as one biological replication. Initially, all plants were grown under the control temperature regime in the phytotron chamber. Starting from one week after crop emergence, the plants were watered every 2-3 days based on the growth stage and water use. Once a week, a quick release fertilizer (20

N:20 P_2O_5:20 K_2O) prepared at a concentration of 3 g L^{-1} was applied at a rate of 100 ml per pot starting one week after emergence. At the flowering stage II-III when plants developed the first flower bud prior to anther dehiscence (Jiang et al., 2019), pots of all varieties in the heat treatment group were transferred from the control temperature regime to the high temperature regime for the desired HS, i.e., 3 h 38°C. After the heat treatment, all the anthers and stipules on the first flowering node of the three plants within one pot were sampled and then were freshly frozen in liquid nitrogen and kept at -80°C for storage. For the plants in the control temperature group, the anthers and stipules from an individual pot were sampled at the same physiological timing as the HS group mentioned above and were stored at -80°C as well.

4.3.3 RNA extraction and RNA integrity check

The whole experiment constituted a library of 36 samples from three varieties, two plant organs, two temperature treatments and three biological replicates, as detailed in the previous section. For each organ sample, the extraction of total RNA was conducted using Rneasy Plant Mini Kit (QIAGEN Inc, Germany), and then a further clean-up step by digesting any remaining DNA contaminant was carried out using QIAGEN Rnase-free Dnase set. The quantity of extracted RNA sample was then determined by evaluating optical density at 260 nm and the OD260/OD280 absorption ratio using NanoDrop 8000 UV spectrophotometer. The integrity of all 36 RNA samples were profiled for the integrity via Bioanalyzer 2100 according to the manufacturer's manual, and all RNA samples had integrity scores in the range of 9-10 on the scale of 0–10, which passed the integrity standard for sequencing.

4.3.4 RNA-Seq protocol

Construction of cDNA libraries and subsequent sequencing was done at MedGenome Inc (https://www.medgenome.com, Foster City, CA, USA).

4.3.5 Raw data processing and sequencing read alignment

All raw sequence reads were deposited at the NCBI Sequence Read Archive (Bioproject ID: PRJNA757773, BioSample IDs: SAMN20980394 to SAMN20980429). In the pre-processing step of the raw reads, the adapter sequences and low-quality bases were trimmed using AdpaterRemoval-V2 (Schubert et al., 2016). From the preprocessed reads, ribosomal RNA sequences were removed by aligning the reads with SILVA database (Quast et al., 2012) using

Bowtie2_v2.2.9 (Langmead and Salzberg, 2012). The remaining reads were aligned to the pea reference genome (Pisum_sativum_v1a.fa) and gene model (Pisum_sativum_v1a_genes.gff3) (Kreplak et al., 2019). The alignment was preformed using STAR_v2.5.3a (Dobin et al., 2013).

4.3.6 Differential gene expression analysis and annotation

Firstly, a homology search was executed for all 44,756 gene sequences against UniProt plant using Diamond_v0.9.3.104 (Buchfink et al., 2015). Out of 44,756 genes, 33,669 genes were annotated based on a tophit. Then for each variety, the differential expression analysis between heat treatment (3 h 38°C) and control (22°C) was conducted via the cuffdiff program in cufflinks package_v 2.2.1 (Trapnell et al., 2012). Log2 FC cutoff 2/-2 and p-value cutoff 0.01 were used separately as cutoffs for up- and down-regulated genes to characterize DEGs. The unit of measurement used by Cufflinks to estimate transcript abundance is fragments per kilobase of transcript per million mapped reads.

4.3.7 Quantitative real-time PCR validation

To validate the correctness of above DEG results analysed *in silico*, a qPCR bench assay was conducted to test the result consistency between the two methods. Eleven random genes were originally selected from the pea genome and primers were designed for each gene via IDT Primer quest tool (Integrated DNA Technologies Inc) according to the following criteria, i.e., Tm of 62 ± 1°C, PCR amplicon lengths of 90–120 bp, primer length of 20–22 bp, and GC content of 45–55%. A series of 10-time cDNA dilutions on PR11-90_control leaf cDNA library was made for primer efficiency test. And primer efficiency (%) of each gene was equaled to $(10^{-1/slope} - 1) * 100$, and all primers had their efficiency rates between 90–110% and qualified for assay use (Appendix B).

Subsequently, the relative expression of the 11 genes was separately quantified among 18 stipule samples and the 18 anther samples, which were used for RNA sequencing. SensiFAST SYBR No-ROX kit was used for the target gene expression using optical 384 well plate on BIO-RAD CFX384 real-time PCR machine in accordance with the manufacture's protocols. RT-qPCR data were analyzed according to the comparative $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008), where ΔCt = (Ct of gene of interest – Ct of reference gene). For the reference gene that was used as the internal control in each PCR reaction, *GH720838*, encoding transcription factor

IIA, was selected (Die et al., 2010). The relative gene expression change was compared between qPCR bench assay and RNA-Seq via correlation analysis on stipules and anthers separately.

4.3.8 Gene ontology enrichment analyses on heat responsive genes

Further comparative analyses on DEGs were conducted among the three pea varieties with different heat tolerances, and between anther (reproductive plant organ) and stipule (vegetative plant organ). The results were output in Venn diagram via online software (http://bioinformatics.psb.ugent.be/webtools/Venn/). Subsequently, GO terms of heat responsive genes were tested against the pea reference transcriptome (Pisum_sativum_v1a_GO, database was retrieved in November, 2020) via agriGO v2.0 (Tian et al., 2017), and significant GO terms in biological processes were filtered using hypergeometric test method at FDR adjusted p value < 0.01.

4.3.9 Graphical overview of pea heat stress related metabolism pathways

To visualize the metabolic pathways that were associated with all DEGs of the three varieties in individual plant organs, 'Metabolism overview' function in MapMan software v. 3.6.0 (Usadel et al., 2009) was used for visual presentation on HS related metabolic responses of anther and stipule separately.

4.4 Results

4.4.1 Sequencing quality assessment

To understand transcriptional reprogramming of field pea in response to HS, we performed deep RNA sequencing of stipule and anther organs subjected to 38°C for 3 h among three varieties using the NovoSeq sequencing platform. The sequencing platform produced a high confidence sequencing output with a < 2% maximum read error rate among these 36 libraries. After removing the error reads, the anther libraries had an average of 84 million 100 bp paired-end reads across the three varieties (Table 4.2). Stipule libraries resulted in a similar average of 88 million reads (Table 4.3). The high sequencing depth of both plant organs provides sufficient resolution for the global transcriptome analysis as compared with previous pea transcriptome studies (Alves-Carvalho et al., 2015; Chen et al., 2013; Sudheesh et al., 2015). Subsequently the reads were mapped to the pea reference genome (Kreplak et al., 2019), and

nearly all reads were successfully aligned to the pea genome, which also implies good quality of the deep sequencing.

Table 4.2. Summary of sequencing depth and percentage of sequencing reads aligning to pea genome on anther samples.

Genotype	Rep	Treatment	No. of million reads	Alignment (%)
CDC Amarillo	1	22°C control	65.2	99.6
CDC Amarillo	2	22°C control	100.1	99.7
CDC Amarillo	3	22°C control	88.3	99.7
CDC Amarillo	1	38°C stressed	65.0	99.2
CDC Amarillo	2	38°C stressed	88.1	99.2
CDC Amarillo	3	38°C stressed	87.4	99.6
PR11-2	1	22°C control	74.4	99.7
PR11-2	2	22°C control	75.9	99.7
PR11-2	3	22°C control	94.6	99.8
PR11-2	1	38°C stressed	83.8	99.1
PR11-2	2	38°C stressed	82.1	99.0
PR11-2	3	38°C stressed	80.8	99.2
PR11-90	1	22°C control	88.6	99.6
PR11-90	2	22°C control	80.2	99.7
PR11-90	3	22°C control	98.0	99.7
PR11-90	1	38°C stressed	85.0	99.6
PR11-90	2	38°C stressed	85.7	99.4
PR11-90	3	38°C stressed	90.9	99.4

Table 4.3. Summary of sequencing depth and percentage of sequencing reads aligning with pea genome on stipule samples.

Genotype	Rep	Treatment	No. of million reads	Alignment (%)
CDC Amarillo	1	22°C control	101.7	99.3
CDC Amarillo	2	22°C control	92.1	99.5
CDC Amarillo	3	22°C control	100.1	99.5
CDC Amarillo	1	38°C stressed	82.6	97.3
CDC Amarillo	2	38°C stressed	78.3	98.4
CDC Amarillo	3	38°C stressed	80.4	99.1
PR11-2	1	22°C control	90.3	99.6
PR11-2	2	22°C control	90.4	99.3
PR11-2	3	22°C control	87.9	99.6
PR11-2	1	38°C stressed	85.2	98.8
PR11-2	2	38°C stressed	84.7	99.3
PR11-2	3	38°C stressed	106.4	99.3
PR11-90	1	22°C control	77.5	99.0
PR11-90	2	22°C control	109.8	99.6
PR11-90	3	22°C control	80.4	99.5
PR11-90	1	38°C stressed	74.7	98.9
PR11-90	2	38°C stressed	66.5	99.0
PR11-90	3	38°C stressed	107.7	99.3

4.4.2 Validation of the characterized heat responsive genes *in silico*

The stipule expression response (log2 FC) of the 11 randomly selected genes between the heat treatment and the control temperature were characterized via cuffdiff program and qPCR respectively, and the results are shown in a heat map. Nine genes out of the eleven, had consistent HSRs between qPCR and RNA-Seq results *in silico* (Fig 4.1), implying a good quality of RNA-Seq analysis. The appropriateness of DEG characterization in RNA-Seq was further confirmed by the significant correlation with qPCR results ($R^2 = 0.97$, Fig 4.2a). Two genes had some unmatched results between the two methods. 0s3930g0040 displayed a consistently up-regulated expression via qPCR among the three pea varieties when subjected to heat treatment (Fig 4.1), whereas for the *in silico* result only CDC Amarillo had the same trend. From *in silico* result, 5g006560 demonstrated a consistent downregulation towards HS in all varieties, whereas in qPCR result, only PR11-90 had the similar trend. Likewise, among anther samples, a high

consistency was found between bench results and cuffdiff results (Figs 4.1 and 4.2b). Unmatched results were mainly observed on gene 5g006560. The significantly high correlation ($R^2 = 0.93$) between the two methods confirmed the correctness of the analyses. The qPCR results successfully validated the correctness of RNA-Seq analysis on both anther and stipule samples, as a result, the DEG lists were utilized for GO enrichment analyses in the following sections.

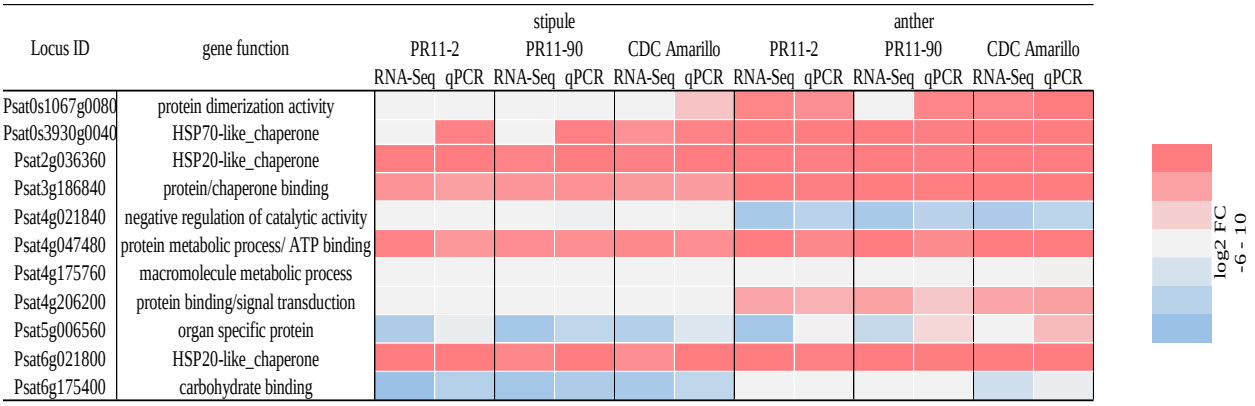


Fig 4.1. Transcriptional heat stress response heatmap of 11 randomly selected genes in the pea genome via qPCR and cuffdiff *in silico* methods. Fold change values are the average log2 FC across three biological replicates; the red color is for upregulation and the blue color is for downregulation.

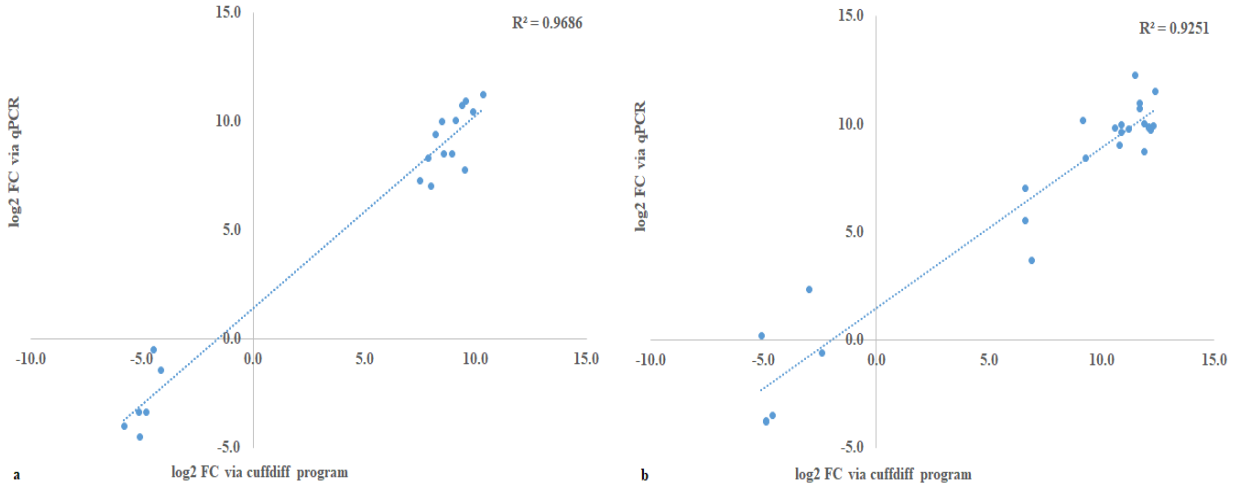


Fig 4.2. Gene expression result correlation on stipule samples (panel a) and anther samples (panel b) between qPCR and cuffdiff program.

4.4.3 Global comparisons of heat stress related transcriptomes between stipules and anthers among three pea varieties

To gain the knowledge on gene response to HS, genes whose expression differed between HS and control temperature at log2 $|FC| \geq 2$ were characterized as heat responsive genes. A total of 3565 responsive genes were identified in anthers, among which 2322 genes had greater expression and 1243 had lower expression in heat treatment compared to control temperature (Fig 4.3a, 4.3d). Stipules on the same flowering node had 4381 responsive genes, with 1886 up-regulated genes and 2495 down-regulated genes (Fig 4.3b, 4.3e). Among anther transcriptomes of the three varieties, the number of genes that were up-regulated under HS was almost twice the number of down-regulated genes. The three varieties shared 588 genes with up-regulated expression under HS, which comprised of 25% up-regulated genes in total. The overlap between PR11-2 and PR11-90, where the two varieties were derived from the same recombinant inbred population, accounted for a higher proportion (~70% in PR11-2 and ~60% in PR11-90). CDC Amarillo, which has a different genetic background, contributed a major group of DEGs that were distinctly up-regulated. Among the 1243 genes whose expression was inhibited, 220 genes were found common among the three varieties.

Whereas among the surrounding stipule leaf transcriptomes, the pattern was opposite compared to the anther transcriptome, i.e., a greater number of genes were down-regulated in the heat treatment. The result revealed a different HSR in stipules compared to anthers. Still, there were common DEGs between anthers and stipules, 221 common DEGs with their up-regulated expression and 25 DEGs with down-regulated expression (Fig 4.3c, 4.3f). Respective GO enrichment analysis of the two groups of DEGs was conducted to cluster their functions in plant biological processes, and results were elucidated in the section below on GO analysis.

Among the three varieties, PR11-2, considered to be best heat tolerant, had the lowest number of its DEGs in both anthers and stipules, indicating that PR11-2 might be able to maintain a relatively steady transcriptome when subjected to a short term HS. In anthers, PR11-90 had a similar number of total DEGs as that of CDC Amarillo, but CDC Amarillo had a greater number of up-regulated genes and lower number of down-regulated genes than PR11-90. Whereas in stipules, CDC Amarillo had both higher numbers of up-regulated and down-regulated genes than PR11-90. It is worth noting that CDC Amarillo appeared to have more

unique DEGs in HSR compared with the other two varieties whose genetic backgrounds were more similar. This finding implied that HSR could depend on genetic variability. In the above comparative analysis, the set of genes that were common between the organs across varieties can be considered as genes associated with general HSR in pea. Both organ-specific and variety-specific DEGs from the comparative analysis could aid in deciphering the genetic basis of heat tolerance and be useful in marker-assisted breeding of heat tolerant pea varieties. Consequently, GO enrichment analyses on separate DEGs in these two aspects were conducted in the following section.

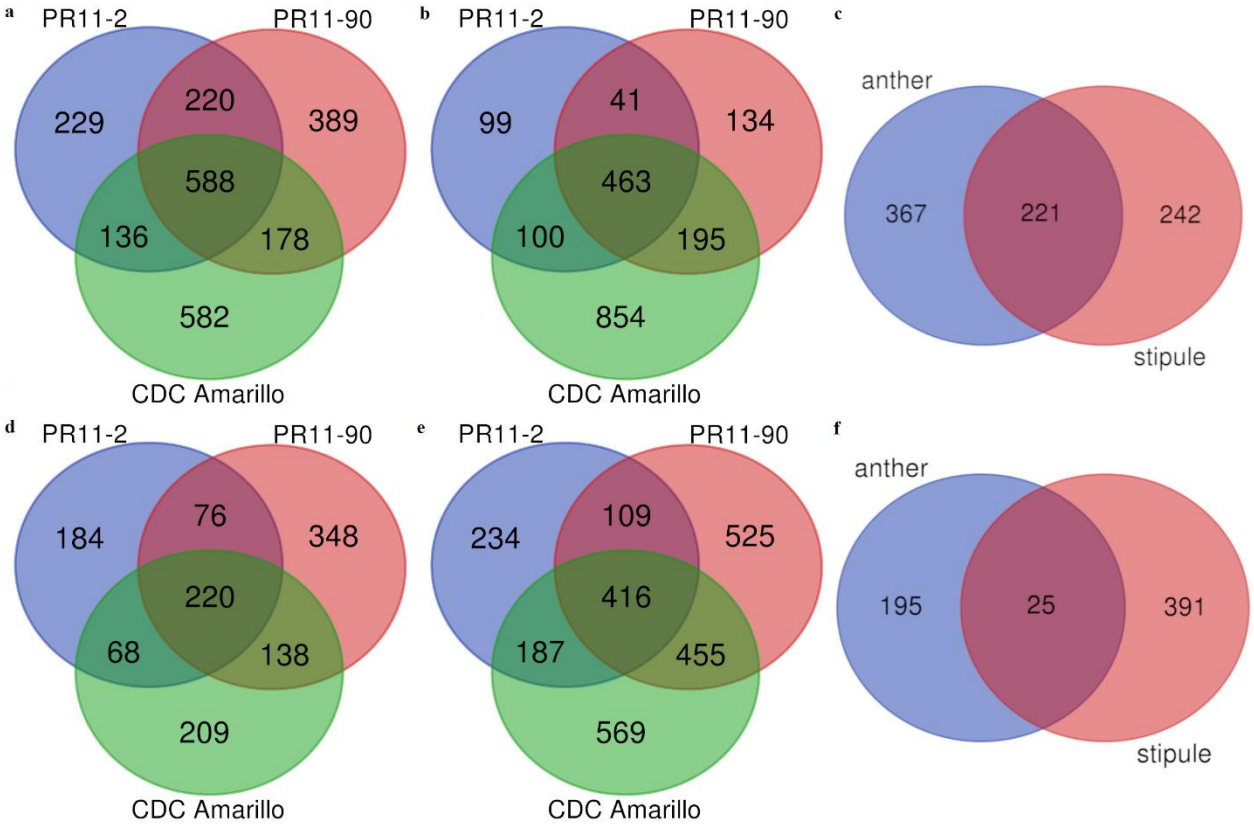


Fig 4.3. Venn diagram showing the number of common and specific heat responsive genes (log2 |fold change| ≥ 2; false discovery rate < 0.05) at 3 h 38°C heat treatment among three pea varieties, and between anther and stipule on the same node. Panel a-c are for up-regulated genes (from the left to right are anther, stipule and comparison between the two). Panel d-f are for down-regulated genes in the same order mentioned above.

4.4.4 Gene ontology grouping on common heat responsive genes among pea varieties

With the purpose of characterizing a general pea plant HSR, GO enrichment analysis was conducted on the common DEGs among the three pea varieties in this study. In anthers, GO terms relating to the 588 common up-regulated genes and 220 down-regulated genes (Fig 4.3a, 4.3d) were tested separately against the pea reference transcriptome (Pisum_sativum_v1a_GO, database was retrieved in November, 2020) to identify the significantly over-represented GO terms in biological processes under HS. All significant GO terms were filtered via hypergeometric test method at FDR adjusted p value ≤ 0.01. Respective analyses were similarly conducted on the common genes in stipules, i.e., 463 DEGs with upregulation and 416 DEGs with downregulation (Fig 4.3b, 4.3d). Up-regulated genes were enriched with 31 and 13 GO terms in biological processes for anthers and stipules, respectively (Fig 4.4, Appendix C). The top 10 most significant GO terms in anthers were protein folding (GO:0006457, 21 enriched terms), embryo development (GO:0009790, 9), multicellular organismal process (GO:0032501, 17), response to heat (GO:0009408, 5), multicellular organism development (GO:0007275, 15), galactose metabolic process (GO:0006012, 4), regulation of transcription, DNA-templated (GO:0045449, 43), regulation of cellular metabolic process (GO:0031323, 44), regulation of RNA metabolic process (GO:0051252, 26), and regulation of gene expression (GO:0010468, 44). In the stipules located on the same anther bearing node, the ten most over-represented GO terms were protein folding (GO:0006457, 13), response to heat (GO:0009408, 4), cellular protein modification process (GO:0006464, 12), carbohydrate metabolic process (GO:0005975, 80), post-translational protein modification (GO:0043687, 12), transcription, DNA-templated (GO:0006351, 21), RNA biosynthetic process (GO:0032774, 19), phosphate-containing compound metabolic process (GO:0006796, 11), phosphorus metabolic process (GO:0006793, 11) and regulation of RNA metabolic process (GO:0051252, 18). Interestingly, four GO terms were common between anthers and stipules, which were GO:0006457 (protein folding), GO:0009408 (response to heat), GO:0006351 (transcription, DNA-templated) and GO:0051252 (regulation of RNA metabolic process). GO:0051252 is one of the ancestor terms of GO:0006351 in the cluster. These four biological processes seem strongly associated with the basal heat tolerance of pea. Other enriched GO terms of consistently up-regulated genes in anthers were involved with primary metabolic processes, cellular respiration and reproductive structure development and the regulations of several biosynthetic and metabolic clusters

including cellular metabolic and biosynthetic process, RNA metabolic process, macromolecule metabolic and biosynthetic process (Appendix C).

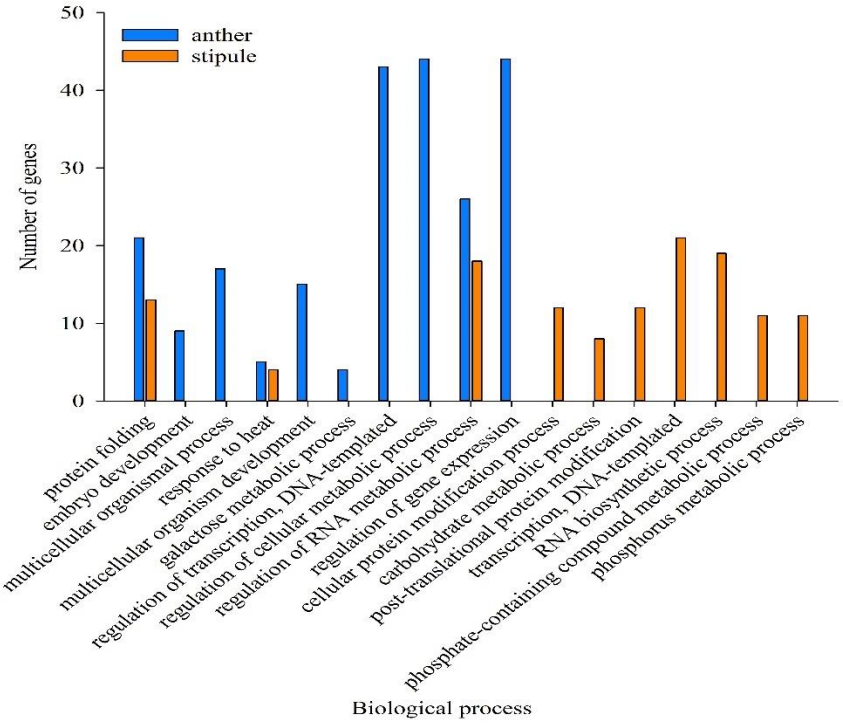


Fig 4.4. Top ten most significantly up-regulated biological processes in response to heat shock in anthers (blue column) and stipules (orange column) among three pea varieties.

To further compare the most heat responsive genes among different varieties, we arbitrarily filtered the DEGs of each variety within the top 20%-fold threshold range and found

the most heat inducible genes were quite similar, though the greatest gene expression fold threshold varied slightly among the three varieties. The gene group relating to HSF and HSP accounted for a large proportion.

Many of these HSF and HSP genes were reported here for the first time; HSF genes in particular, which expanded the previously limited findings. Putative HSF family A and B genes appeared to heat inducible, these genes included three HSFA genes and two HSF B genes (corresponding gene locus refers to Table 4.4). In addition to the two pea HSP genes that were previously documented, 11 other small HSP (sHSP) genes on chromosomes II, IV, V, VI and VII were highly heat inducible among all varieties regardless of plant organs. Increased expression of six HSP70 genes, two HSP90 family genes and three other HSP genes were also identified among all three varieties. Several heat shock cognate genes (HSCs), whose expression was previously considered as constitutive during normal plant development, appeared to be heat responsive as well (e.g., PsHSC71.0, HSC70-2 like etc). The response of several other genes whose functions closely interacted with HSP were also detected in this study.

Interestingly, several HSP genes only responded in one organ. Anthers had unique HSFs (Psat0s3914g0040, putative HSF; Psat4g086800, HSF24-like), three HSP genes which were Psat1g222760 (Stromal HSP70), Psat3g104360 (HSP83-like fragment), Psat5g229840 (class IV HSP). Stipules had one unique HSF (Psat6g078240, HSFA3-like) and two HSP genes (Psat7g255520, HSP26.5; Psat3g180040, HSC70-2-like). Several other HSF and HSP genes were specific to variety, e.g., two HSP relating genes (Psat4g003160 and Psat4g035840) were only induced in PR11-90. Because many HSFs and HSPs have been reported here for the first time, our findings are further discussed with corresponding findings in other plant species.

Table 4.4. List of pea heat shock protein and heat shock factor related genes that were induced in response to 3 h 38°C heat treatment; numbers in the table are averaged log2 (fold changes) across three biological replicates.

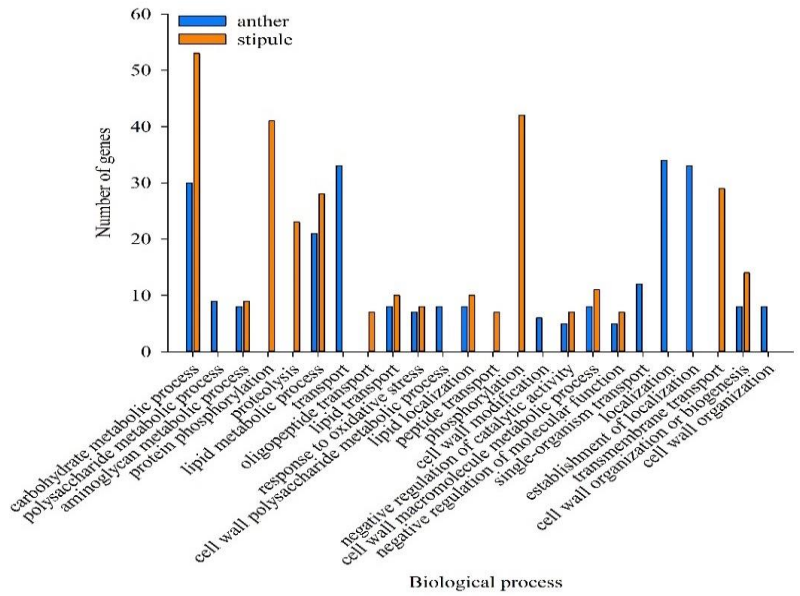
Locus ID	PR11-2		PR11-90		CDC Amarillo		Gene annotation
	anther	stipule	anther	stipule	anther	stipule	
Psat0s1635g0080	8.8	8.1	9.0	8.7	8.2	8.0	PsHSP18.1
Psat2g036480	10.8	10.4	12.5	9.3	11.4	9.1	PsHSP17.9 fragment
Psat0s3930g0040	10.9		12.1		11.7	8.2	PsHSP71.2
Psat3g049640	2.8		2.9	2.3	2.9	2.3	PsHSC71.0
Psat0s3914g0040			2.5		2.4		putative HSF
Psat1g102600	2.0	3.9	2.1	4.0		5.4	HSFB-2A-like
Psat2g021040	inf		inf	2.4			putative HSF
Psat3g061600	9.3	7.8	9.7	7.8		7.5	HSFA3
Psat4g086800	inf		inf		inf		HSF24-like
Psat5g036400	7.7	7.4	9.2	8.1	7.1	7.5	HSFA fragment
Psat6g059040	10.1	6.1	9.8	6.8	8.7	6.2	putative HSFA3
Psat6g078240		5.7		5.8		6.9	HSFA3-like protein
Psat6g200480	5.8	7.1	7.2	7.9	5.6	8.1	HSFB2A-like isoform X2
Psat6g204120				3.2		2.5	putative HSFA3
Psat7g004560		2.0				2.7	putative HSF
Psat7g131680						2.4	putative HSF
Psat7g170680						3.6	HSFA1B-like isoform X2
Psat0s529g0040	10.1	10.0	12.5	9.0	12.6	9.0	putative class I HSP20
Psat2g046440	6.3	6.6	8.2	8.2	6.2	7.8	HSP15.7 peroxisomal-like
Psat4g136720	7.3	7.4	8.1	8.5	7.0	7.4	sHSP
Psat5g035320	10.5	10.3	11.9	9.3	12.2	9.3	class II HSP17.1
Psat4g166400	10.3	9.9	12.3	8.9	13.0	9.5	cytosolic class II HSP
Psat5g073280	11.7	10.5	13.1	9.8	11.1	8.9	sHSP
Psat5g174800	9.1	7.4	11.0	6.9	8.5	7.1	putative HSP20
Psat6g112800	10.8	10.0	13.0	9.4	11.8	9.0	class IV HSP22.7
Psat7g114760	7.5	9.9	11.3	9.0	11.5	11.9	class I HSP17.6
Psat7g115480	7.1	9.0	7.6	9.3	6.7	8.4	HSP18.1

Psat7g211720	5.4	6.4	6.0	6.9	4.5	6.2	class I HSP17.5
Psat7g255520		2.8		3.2		3.6	HSP26.5
Psat6g238960					2.3		DnaJ/HSP40 cysteine-rich domain protein
Psat1g212880	7.1	4.9	8.1	5.7	7.1	6.5	HSP70, mitochondrial
Psat1g222760	4.9		5.2		4.5		Stromal HSP70
Psat2g051360	8.9	6.5	10.4	6.6	8.4	7.2	HSP70
Psat3g143400		2.5	2.1	2.6		3.5	HSP70-interacting protein
Psat3g180040		2.0		2.4		2.2	HSC70 2-like
Psat3g183720	5.1	5.9	5.4	6.5	4.6	6.1	putative HSP70 family
Psat4g003160			2.1				HSP70-interacting protein
Psat4g035840				2.2			HSP70 8-like
Psat4g210520	inf	3.9	6.5	4.2	5.4	5.0	HSP70
Psat5g299000	3.8	2.9	4.3	3.1	3.6	3.8	putative HSP70 family
Psat7g023360	4.5	4.3	5.2	4.9	4.5	4.8	HSP70
Psat7g218840	8.9	7.7	10.0	8.3	8.6	7.8	HSC70 2-like
Psat7g237280	4.7	4.4	5.1	4.9	4.7	5.3	HSP70 nucleotide exchange factor FES1-like
Psat2g006440	5.4	4.6	5.7	5.4	5.2	5.4	HSP81-2
Psat0ss29864g0040	10.8	10.2	12.1	9.1	13.0	9.2	HSP83-like fragment
Psat3g104360	8.5		9.9		8.3		HSP83-like fragment
Psat5g164840	2.6	2.0	3.0	2.8	2.5	2.8	HSP80 cognate protein
Psat6g123080	4.2	3.8	4.7	4.2	3.9	4.9	activator of HSP90 ATPase homolog 1-like
Psat2g178800	5.0	3.8	5.5	4.0	4.9	4.6	HSP70-HSP90 organizing protein 3-like
Psat3g067000	5.2	3.9	5.7	4.4	4.9	5.2	activator of HSP90 ATPase homolog 1
Psat0s3618g0080	2.4	3.4	3.0	4.0	2.2	5.2	HSP DnaJ; putative transcription factor C2H2 family
Psat1g204360	10.3	10.2	11.7	9.2	12.5	9.4	HSP DnaJ
Psat2g037160	3.3						HSP
Psat5g035280	10.7	10.4	12.0	9.4	10.9	9.2	Class II HSP
Psat5g229840	3.8		3.7		2.3		Class IV HSP
Psat6g021840	9.5	8.2	11.1	8.8	10.4	7.5	Class II HSP

Psat7g114360	10.6	10.2	12.5	9.4	10.9	9.0	HSP

Note: for the cells denoting 'inf' as fold change, the reason was that their transcripts at control temperature were too low to quantify. Because their transcripts at heat stress were significant, they were still considered as heat responsive.

A total of 220 commonly down-regulated genes in anthers among the three varieties (Fig 4.3d) were enriched in 18 GO terms of biological process category and 416 consistently down-regulated genes in stipules (Fig 4.3e) had 16 GO terms significantly over-represented (Fig 4.5). Ten GO terms overlapped between the two organ types, that is, GO:0006629 (lipid metabolic process), GO:0006869 (lipid transport), GO:0010876 (lipid localization), GO:0044036 (cell wall macromolecule metabolic process), GO:0071554 (cell wall organization or biogenesis), GO:0006979 (response to oxidative stress), GO:0005975 (carbohydrate metabolic process), GO:0006022 (aminoglycan metabolic process), GO:0043086 (negative regulation of catalytic activity), and GO:0044092 (negative regulation of molecular function). These ten GOs represented the ten biological processes that were generally HS damaged in pea plants, as genes in these ten biological processes were significantly down-regulated in both vegetative and reproductive pea organs. GO:0006508 (proteolysis), GO:0006468 (protein phosphorylation), GO:0015833 (peptide transport) and GO:0006857 (oligopeptide transport) were distinctly enriched in stipule down-regulated genes, whereas GO:0005976 (polysaccharide metabolic process), GO:0010383 (cell wall polysaccharide metabolic process), GO:0042545 (cell wall modification) and GO:0071555 (cell wall organization) were only enriched in anthers. Although more than half of the over-represented GO terms overlapped between heat stressed pea anthers and stipules at the same flowering node, surprisingly, the gene composition relating to these biological processes varied between the two organs. For example, three GO terms related to lipid biological processes were both down-regulated in anthers and stipules. However, only two genes (*PsLTP1* and *PsLTP2*) for lipid transport/localization were common, and seven genes (Psat1g060840, Psat1g082320, Psat1g085080, Psat2g027880, Psat3g005680, Psat5g104040, and Psat5g295040) for lipid metabolic process were common between the two organ types (Table 4.5). It was apparent that genes in the lipid biological processes were spatially HS regulated.



491

492 Fig 4.5. Significantly down-regulated biological processes (FDR adjusted p value at 0.01) in
493 anthers (blue column) and stipules (orange column) of three pea varieties.

Table 4.5. Gene locus and function list of commonly down-regulated genes that are associated with lipid transport, localization and metabolic process among the three pea varieties in anthers and stipules.

GO:0006869 lipid transport/ GO:0010876 lipid localization			
anther		stipule	
locus ID	gene function	locus ID	gene function
Psat0s1251g0040	Non-specific lipid-transfer protein	Psat0s2857g0040	Lipid transfer protein
Psat0s4118g0160	Non-specific lipid-transfer protein	Psat1g217760	Non-specific lipid-transfer protein
Psat3g119520	Non-specific lipid-transfer protein	Psat3g097600	Putative non-specific lipid-transfer protein AKCS9-like protein
Psat3g119560	Non-specific lipid-transfer protein	Psat5g029400	lipid transfer protein EARLI 1-like
Psat7g233960	Non-specific lipid-transfer protein	Psat5g112720	Lipid transfer protein
Psat7g234520	Non-specific lipid-transfer protein 2 (PsLTP2)	Psat6g027760	14 k Da proline-rich protein DC2.15-like
Psat7g234640	Non-specific lipid-transfer protein 2 (PsLTP2)	Psat7g226840	Non-specific lipid-transfer protein
Psat7g234720	Non-specific lipid-transfer protein 3 (PsLTP1)	Psat7g228160	Non-specific lipid-transfer protein 1 (LTP1)
		Psat7g234680	Non-specific lipid-transfer protein 2 (PsLTP2)
		Psat7g234720	Non-specific lipid-transfer protein 3 (PsLTP1)
GO:0006629 lipid metabolic process			
anther		stipule	
locus ID	gene function	locus ID	gene function
Psat0s1560g0040	GDSL esterase/lipase	Psat0s1401g0160	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)
Psat1g060840	Pathogen-inducible alpha-dioxygenase	Psat0s1926g0240	Auxilin-like protein (Fragment)
Psat1g081400	uncharacterized protein LOC101505667 isoform	Psat0s2010g0040	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)
Psat1g082320	GDSL esterase/lipase	Psat0s3211g0160	GDSL esterase/lipase (Fragment)
Psat1g085080	GDSL esterase/lipase LTL1-like	Psat1g017360	Fungal proteinase A aspartic proteinase superfamily protein
Psat1g086280	Lipase	Psat1g060840	Pathogen-inducible alpha-dioxygenase
Psat1g096440	3-ketoacyl-CoA synthase-like protein	Psat1g082320	GDSL esterase/lipase
Psat1g200800	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)	Psat1g085080	GDSL esterase/lipase LTL1-like
Psat2g027880	Uncharacterized protein	Psat1g193000	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)
Psat3g005680	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)	Psat2g027800	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)
Psat3g006280	GDSL esterase/lipase	Psat2g027880	Uncharacterized protein

Psat5g104040	GDSL esterase/lipase At2g04570-like	Psat2g083600	3-ketoacyl-CoA synthase (EC 2.3.1.-)
Psat5g284160	3-ketoacyl-CoA synthase (EC 2.3.1.-)	Psat2g132440	PI-PLC X domain-containing protein At5g67130
Psat5g295040	GDSL esterase/lipase apg-like protein	Psat3g000920	3-ketoacyl-CoA synthase (EC 2.3.1.-)
Psat6g041080	GDSL-like lipase/acyl hydrolase	Psat3g005640	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)
Psat6g184320	Patatin (EC 3.1.1.-)	Psat3g005680	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)
Psat7g066400	GDSL esterase/lipase	Psat3g010160	Uncharacterized protein
Psat7g066440	GDSL-like lipase/acyl hydrolase	Psat4g010320	Fatty acid hydroxylase protein (EC 4.1.99.5)
Psat7g066520	GDSL-like lipase/acyl hydrolase	Psat4g020480	cyprosin-like
Psat7g125680	PLC-like phosphodiesterase superfamily protein	Psat4g190160	Phospholipase D alpha
		Psat4g196720	GDSL esterase/lipase apg-like protein
		Psat5g104040	GDSL esterase/lipase At2g04570-like
		Psat5g177200	GDSL-like lipase/acyl hydrolase (EC 3.1.1.3)
		Psat5g295040	GDSL esterase/lipase apg-like protein
		Psat6g002160	GDSL-like lipase/acyl hydrolase
		Psat7g059400	GDSL-like lipase/acyl hydrolase (EC 3.2.1.51)

Note: for the genes in the list, their transcription level between the heat treatment and the control temperature was at log2 (fold change) ≤ -2.

Arbitrarily, DEGs of each variety was filtered within the top 20%-fold threshold range to further characterize a list of genes whose functions were most inhibited in HS. In anthers, 35 genes were shared among the three varieties, among which seven genes were involved in pectin metabolism, and three with lipid metabolism. Pectin, a polysaccharide polymer of galacturonic acid with different degrees of esterification via an a-1, 4-glycosidic bond, is a primary composition in the plant cell wall and cell interlayer. In stipules, 51 genes were common among the three varieties. The functions of these genes seemed various, including four lipase genes.

4.4.5 Gene ontology analysis on variety-dependant heat responsive genes

To compare HSRs among the three pea varieties, individual GO enrichment analyses were performed on the distinct DEGs of each variety, which were exclusive DEGs from the varieties' common DEGs in individual variety DEG list. Among the three varieties, PR11-2 had the lowest number of enriched GO terms in down-regulated genes and the highest number of over-representative GO terms in up-regulated genes of both anthers and stipules, implying that PR11-2 is likely to have a superior heat tolerance compared to the other two varieties (Fig 4.6). In the anther transcriptome of PR11-2, no GO term was significantly enriched for down-regulated DEGs but 13 terms were up-regulated. These terms corresponded to four biological pathways, i.e., cell respiratory electron transport chain (GO:0022904), cell wall lignin metabolic and catabolic process (GO:0009808, GO:0046274), oxidation-reduction process (GO:0055114), and cellular modified amino acid catabolic process (GO:0042219). In contrast, the up-regulated GO terms in its stipule were related to regulation of transcription (GO:0006355), DNA repair (GO:0006281), and response to hormone (GO:0009725).

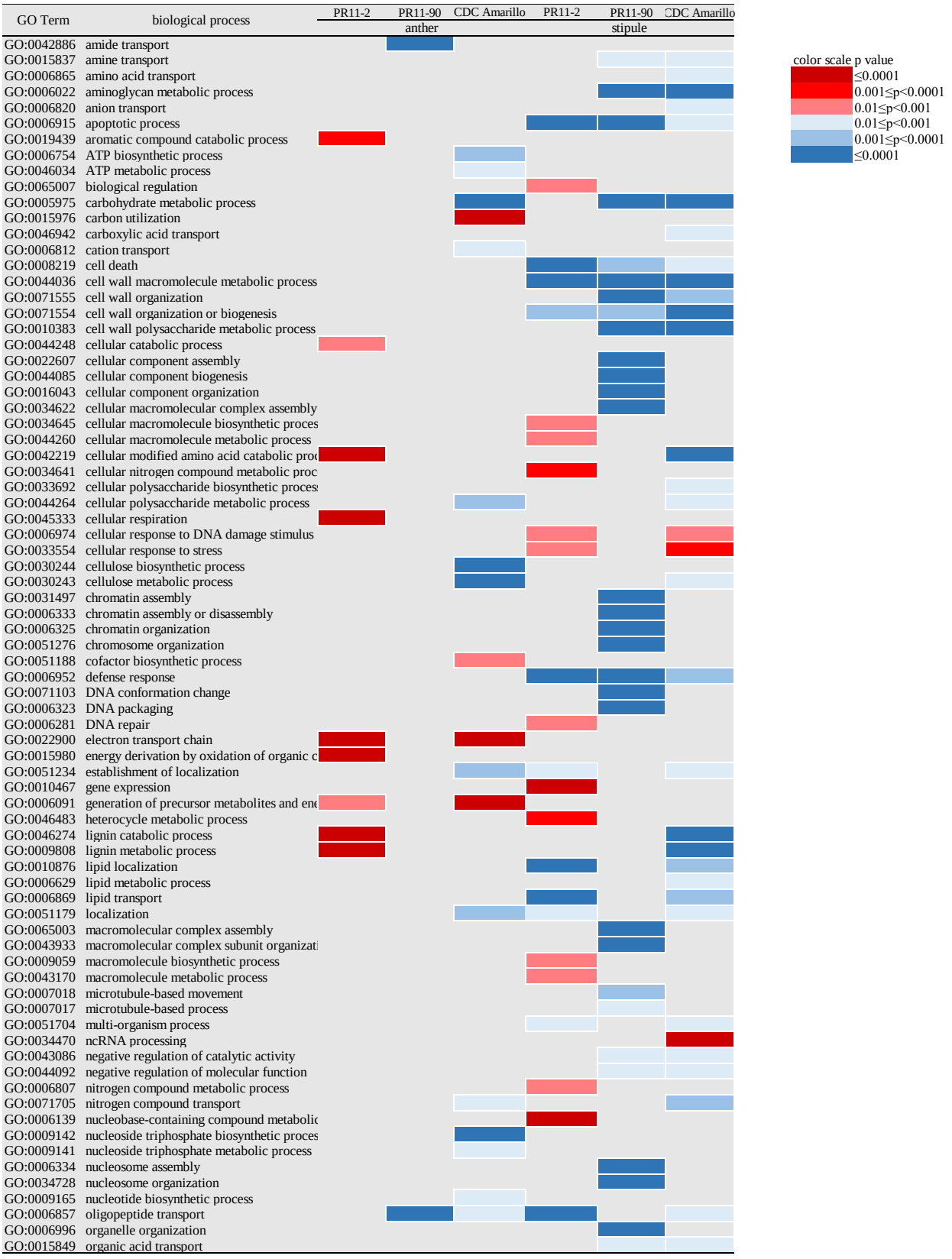
PR11-90 (heat susceptible) had none and two GOs significantly up-regulated in anthers and stipules, respectively; whereas four and 39 GO terms were down-regulated in anther and stipule. The two up-regulated terms corresponded with response to water (GO:0009415). The four anther down-regulated terms were associated with amide transport (GO:0042886), oligopeptide transport (GO:0006857) and oxidation-reduction process. And the 39 GOs with down-regulation in stipule included 19 terms in the cluster of nucleosome assembly (GO:0006334), two terms in microtubule-based movement (GO:0007018) and response to auxin (GO:0009733) that were only over-representative in PR11-90. These distinctly heat prohibited processes in PR11-90 were predicted to link with its heat susceptible property.

In both anther and stipule transcriptomes of CDC Amarillo, the number of down-regulated GO terms was also greater than that of GO terms with upregulation (29/12 downregulation/upregulation in anther; 46/9 downregulation/upregulation in stipule) and had the highest total number of GOs in both anthers and stipules among the three pea varieties. This differential of heat responsive GOs among the three varieties demonstrated the genetic variation of field pea in HSR and shed a light in deciphering molecular mechanism involved in pea heat response and heat tolerance. In anthers, the significantly enriched GO terms in transcriptionally inhibited genes consisted of many GOs in the cluster ATP biosynthetic (GO:0006754) and metabolic (GO:0046034) processes, which were uniquely observed in CDC Amarillo. The 12 enriched terms of genes, whose expression was induced in anthers of CDC Amarillo, were associated with rRNA processing (GO:0006364), response to zinc ion (GO:0010043), electron transport chain (GO:0022900) and carbon utilization (GO:0015976). rRNA processing was also up-regulated in stipule in addition to cellular response to DNA damage stimulus (GO:0006974) and protein folding (GO:0006457). The stipule down-regulated GO terms were mainly linked with amino acid transport (GO:00068650), cell wall polysaccharide metabolic process (GO:0010383), lignin metabolic and catabolic process, lipid transport (GO:0006869), lipid localization (GO:0010876), lipid metabolic process (GO:0006629) and protein phosphorylation (GO:0006468).

In stipules, seven GO terms were down-regulated in all three varieties and were involved in apoptotic process (GO:0006915), defense response (GO:0006952) cell wall macromolecule metabolic process (GO:0044036) and polysaccharide metabolic process (GO:0005976). Cell wall polysaccharide metabolic process, plant-type cell wall organization (GO:0009664), photosynthesis, light harvesting (GO:0009765), amine transport (GO:0015837) and aminoglycan metabolic process (GO:0006022) were down-regulated in PR11-90 and CDC Amarillo. Lipid transport and localization were down-regulated in PR11-2 and CDC Amarillo. From GO enrichment analyses, there was not a biological process that was commonly down-regulated in PR11-2 and PR11-90, suggesting the contrasting heat tolerance between the two varieties, which was also seen in field trials.

It is noted that cellular response to stress (GO:0033554) and cellular response to DNA damage stimulus (GO:0006974) was only up-regulated in the stipules of two heat-tolerant varieties, PR11-2 and CDC Amarillo. Comparing DEGs between the two varieties, transcripts of

four genome loci were common, which were Psat2g148040, Psat5g135640, Psat6g105320 and Psat6g199840. In anthers, generation of precursor metabolites and energy (GO:0006091) and electron transport chain (GO:0022900) were only up-regulated in PR11-2 and CDC Amarillo as well, and the transcriptional level of three relating genes (Psat1g132320, Psat1g132440, and Psat6g041400) was induced in both varieties. Interestingly, contrasting response was observed in oxidation-reduction process (GO:0055114) among the anthers' transcriptomes of the three varieties. This biological process was enriched in the up-regulated genes in PR11-2, but significant in the down-regulated genes in PR11-90 and were over-representative in both down/up-regulated genes in CDC Amarillo. Among these genes, eight genes were commonly up-regulated in PR11-2 and CDC Amarillo, 28 genes were down-regulated in CDC Amarillo and PR11-90 (Appendix D). The above biological processes that were only up-regulated in heat tolerant varieties, are believed to enhance heat tolerance in pea, and are worth discussion with the corresponding findings previously covered in other plant species.


GO Term
biological process
PR11-2
PR11-90
CDC Amarillo
PR11-2
PR11-90
CDC Amarillo
anther
stipule
GO:0042886 amide transport
GO:0015837 amine transport
GO:0006865 amino acid transport
GO:0006022 aminoglycan metabolic process
GO:0006820 anion transport
GO:0006915 apoptotic process
GO:0019439 aromatic compound catabolic process
GO:0006754 ATP biosynthetic process
GO:0046034 ATP metabolic process
GO:0065007 biological regulation
GO:0005975 carbohydrate metabolic process
GO:0015976 carbon utilization
GO:0046942 carboxylic acid transport
GO:0006812 cation transport
GO:0008219 cell death
GO:0044036 cell wall macromolecule metabolic process
GO:0071555 cell wall organization
GO:0071554 cell wall organization or biogenesis
GO:0010383 cell wall polysaccharide metabolic process
GO:0044248 cellular catabolic process
GO:0022607 cellular component assembly
GO:0044085 cellular component biogenesis
GO:0016043 cellular component organization
GO:0034622 cellular macromolecular complex assembly
GO:0034645 cellular macromolecule biosynthetic proces
GO:0044260 cellular macromolecule metabolic process
GO:0042219 cellular modified amino acid catabolic proc
GO:0034641 cellular nitrogen compound metabolic proc
GO:0033692 cellular polysaccharide biosynthetic proces
GO:0044264 cellular polysaccharide metabolic process
GO:0045333 cellular respiration
GO:0006974 cellular response to DNA damage stimulus
GO:0033554 cellular response to stress
GO:0030244 cellulose biosynthetic process
GO:0030243 cellulose metabolic process
GO:0031497 chromatin assembly
GO:0006333 chromatin assembly or disassembly
GO:0006325 chromatin organization
GO:0051276 chromosome organization
GO:0051188 cofactor biosynthetic process
GO:0006952 defense response
GO:0071103 DNA conformation change
GO:0006323 DNA packaging
GO:0006281 DNA repair
GO:0022900 electron transport chain
GO:0015980 energy derivation by oxidation of organic c
GO:0051234 establishment of localization
GO:0010467 gene expression
GO:0006091 generation of precursor metabolites and ene
GO:0046483 heterocycle metabolic process
GO:0046274 lignin catabolic process
GO:0009808 lignin metabolic process
GO:0010876 lipid localization
GO:0006629 lipid metabolic process
GO:0006869 lipid transport
GO:0051179 localization
GO:0065003 macromolecular complex assembly
GO:0043933 macromolecular complex subunit organizati
GO:0009059 macromolecule biosynthetic process
GO:0043170 macromolecule metabolic process
GO:0007018 microtubule-based movement
GO:0007017 microtubule-based process
GO:0051704 multi-organism process
GO:0034470 ncRNA processing
GO:0043086 negative regulation of catalytic activity
GO:0044092 negative regulation of molecular function
GO:0006807 nitrogen compound metabolic process
GO:0071705 nitrogen compound transport
GO:0006139 nucleobase-containing compound metabolic
GO:0009142 nucleoside triphosphate biosynthetic proces
GO:0009141 nucleoside triphosphate metabolic process
GO:0006334 nucleosome assembly
GO:0034728 nucleosome organization
GO:0009165 nucleotide biosynthetic process
GO:0006857 oligopeptide transport
GO:0006996 organelle organization
GO:0015849 organic acid transport
color scale p value
≤0.0001
0.001≤p<0.0001
0.01≤p<0.001
0.01≤p<0.001
0.001≤p<0.0001
≤0.0001

572

Fig 4.6. Significant biological processes in response to heat shock in anthers and stipules of individual pea varieties. More intense color means greater significance. The up-regulated biological processes are colored in red, with significant p values of 0.01≤p<0.001, 0.001≤p<0.0001, and ≤0.0001. The down-regulated biological processes are colored in blue, with significant p values of 0.01≤p<0.001, 0.001≤p<0.0001, ≤0.0001.

4.4.6 Visual overview of pea cellular metabolic changes subject to heat stress

With the interest in the global depiction of pea HS-related metabolism changes, individual MapMan analysis of metabolism overview was conducted on anthers and stipules. Multiple metabolic pathways were affected by HS in both plant organs, including cell wall, lipid, carbohydrate and secondary metabolism (Fig 4.7 and Fig 4.8). In GO enrichment analysis, lipid metabolic process (GO:0006629) and cell wall macromolecule metabolic process (GO:0044036) were also significantly down-regulated in anthers and stipules (Fig 4.5), whose importance in HSR was further discussed.

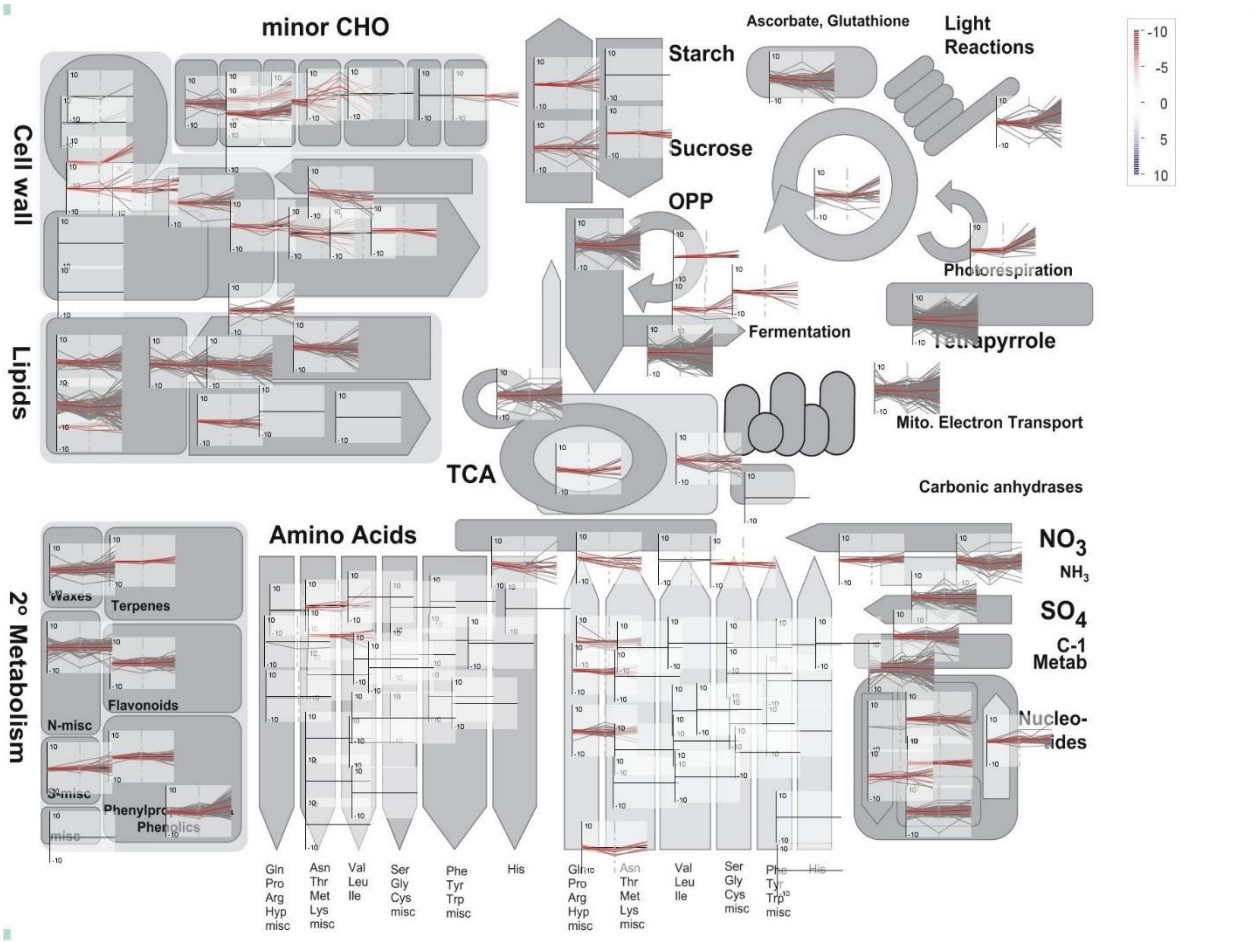


Fig 4.7. MapMan overview of cellular metabolism changes between heat stress (3 h 38°C) and control temperature (22°C) in pea stipules. The colour code scale is based on the log2 fold change; redness represents upregulation and greenness represents downregulation. The grey line in individual axis indicates transcriptional pattern of individual genes across three varieties; thick

red lines represent the average value within all of the clustered genes of individual varieties, and thin red lines represent the average ± one standard deviation.

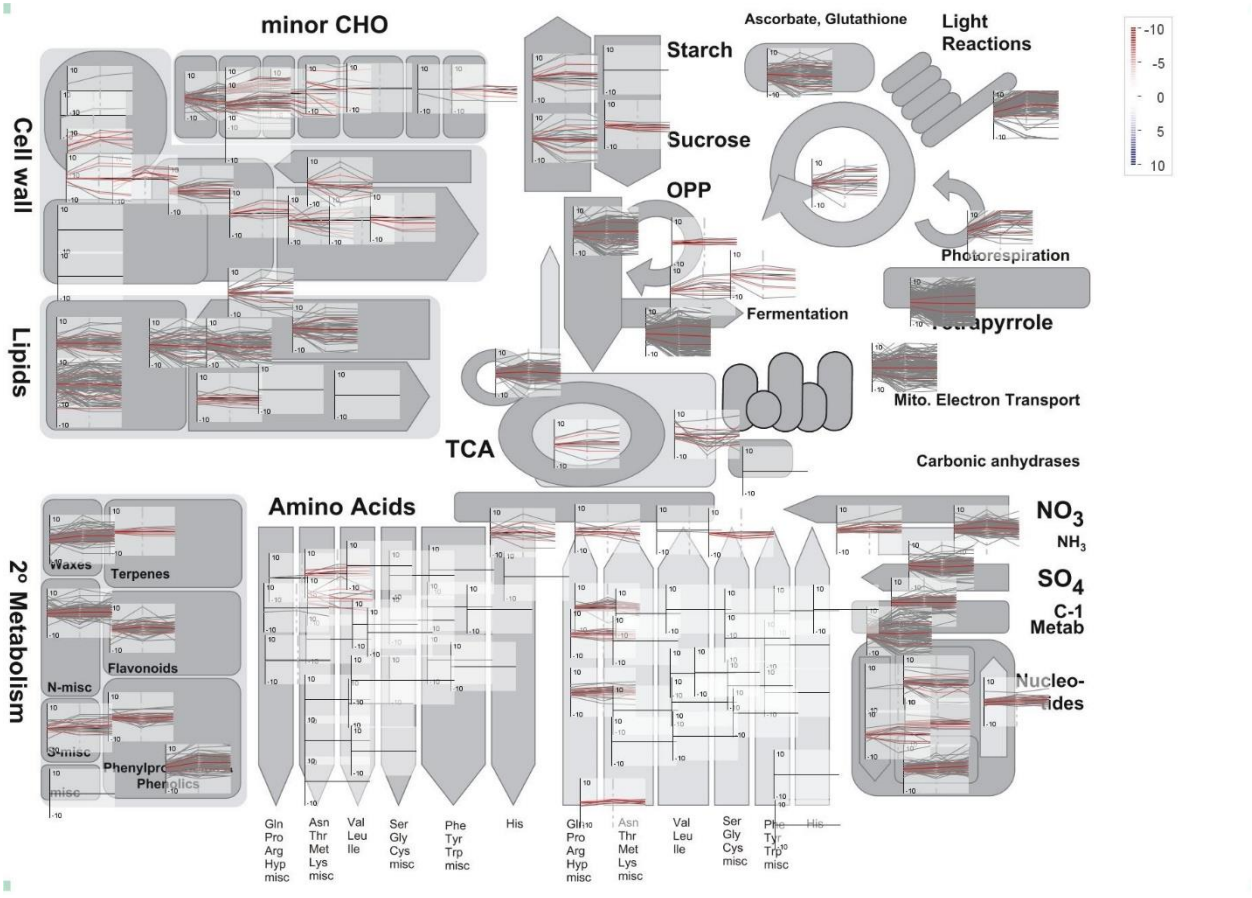


Fig 4.8. MapMan overview of cellular metabolism changes between heat stress (3 h 38°C) and control temperature (22°C) in pea anthers. The colour code scale is based on the log2 fold change; redness represents upregulation and greenness represents downregulation. The grey line in individual axis indicates transcriptional pattern of individual genes across three varieties; thick red lines represent the average value within all of the clustered genes of individual varieties, and thin red lines represent the average ± one standard deviation.

4.5 Discussion

4.5.1 General and genotype specific heat stress responses at the cellular level

Separate heat responsive genes of individual variety were identified at log2 FC ≥ 2 for anthers and stipules, and two heat tolerant varieties, PR11-2 and CDC Amarillo, in our study

607 demonstrated different transcriptomic responses. PR11-2 had the lowest number of DEGs in
608 anther and stipule among the three varieties, contrastingly, DEG number in CDC Amarillo was
609 the greatest in both plant organs (Fig 4.3). This was also seen in maize, where tolerant cultivars
610 S058 and L043 had the most and least abundant DEGs among four tolerant and four susceptible
611 varieties, respectively (Frey et al., 2015). Collectively, it is suggested that plant heat tolerance
612 could be achieve in different mechanisms.

613 Individual GO enrichment analyses were carried out on common DEGs among the three
614 varieties and variety unique DEGs, aiming to characterize the general HSR in biological
615 processes of pea plant as well as unique responses relating to heat tolerance. Response to heat
616 (GO:0009408), protein folding (GO:0006457) and transcription, DNA-templated (GO:0006351
617 and GO:0051252) were commonly upregulated between stipules and anthers (Fig 4.4). The
618 transcriptome re-program and chaperone function of HSPs are considered to contribute to plant's
619 basal thermo-tolerance (Fragkostefanakis et al., 2015). Regressed biological processes were
620 mainly related to lipid transport, lipid metabolic process and cell wall macromolecule metabolic
621 process, and their relevance to HS and relating genes are further discussed in the later section.

622 Regarding variety unique heat response, anther of PR11-2 had only up-regulated
623 processes, belonging to three biological process clusters, i.e., respiratory electron transport chain,
624 lignin catabolic process and cellular modified amino acid catabolic process (Fig 4.6). PR11-90
625 had none induced biological process in anther. This could partly explain heat tolerance of PR11-
626 2 over PR11-90. Intriguingly, electron transport chain (ETC) was also up-regulated in CDC
627 Amarillo. In ETC, Psat1g132320 and 6g041400 encoding mitochondrial cytochrome b and
628 Psat1g132440 encoding uncharacterized protein were up-regulated. Cytochrome b-c1 complex is
629 an essential component of the mitochondrial ETC. Chilling induced accumulation of reactive
630 oxygen species resulting from an over-reduction of ETC led to oxidative stress (Hu et al., 2008).

631 In stipules, cellular response to DNA damage stimulus was only induced in two heat
632 tolerant varieties. Four genes were common between gene lists of the two varieties, which were
633 2g148040 (DNA mismatch repair protein MLH3), 5g135640 (DNA excision repair protein),
634 6g105320 (cryptochrome 2b), and 6g199840 (DNA mismatch repair protein MSH3). The
635 putative functions of the four genes were involved with three DNA repair pathways, but these
636 pathways were well studied in UV light induced stress (Manova and Gruszka, 2015). Elucidation

on the connection of the plant DNA repair to abiotic stress responses remains scarce, plant's ability to maintain its genome integrity is likely to play a role in stress tolerance (Nisa et al., 2019).

4.5.2 Regulatory importance of heat transcription factors A3 and B2 in heat stress response

Although HSFs are believed to play a central regulation role in the transcriptional induction of downstream HS responsive genes, HSFs display their variation in HSR in terms of induction fold threshold and regulation, and thereby could affect various gene expression induction. Structurally plant HSFs are classified into three classes, namely, HSF A, B, and C, based on their structural peculiarities. The best characterized HSF gene family in plants has been firstly reported in Arabidopsis (21 HSF genes; Nover et al., 2001), wheat (56 HSF genes; Xue et al., 2014) and soybean (52 HSF genes; Scharf et al., 2012) were reported to have the largest families in monocot and dicot crops, respectively. Among the three classes, the function of HSFAs was more clearly elucidated, and here is broad agreement that their role most directly leads to heat-induced activation of heat shock genes. HSFA1s are predicted to be the "master regulators" that have the direct role in the activation of transcriptional networks. Knockdown of HSFA1 genes in Arabidopsis led to a reduced induction of many HS-responsive genes, as a result plants demonstrated HS susceptible phenotypes (Liu et al., 2011; Yoshida et al., 2011). The thermo-tolerance conferred by Arabidopsis HSFA1d was further confirmed in a recent study in pea (Shah et al., 2020), where transformant pea plants with this Arabidopsis HSF was more heat tolerant than its wild type due to the increased antioxidant enzyme activity and reduced hydrogen per oxide. Another study in Arabidopsis concluded that HSFA3 was also an important HS-responsive TF, because knockout or knockdown mutations of HSFA3 resulted in a reduced expression of putative target HSP genes during HS (Yoshida et al., 2008). OsHSFA3 and A2s were identified to responsive in rice panicle when exposed to multiple hours of HS (Zhang et al., 2012). In comparison, in common wheat (*Triticum aestivum* L.), HSFA2 and A6 had the highest transcriptional induction among 56 TaHSF members when subjected to HS, which revealed the regulatory importance of these two subclasses during HS (Xue et al., 2014). Among legume plants, an over-expression of soybean *GmHSFA1* could enhance the thermotolerance of transgenic soybeans via the activation of various HSP gene expression (Zhu et al., 2006). In the other study, the induction of GmHSFs at HS was found to variate at different plant stages, including HSFA1 (Soares-Cavalcanti et al., 2012). In *Lotus japonicus*, HSFA1 did not

dominantly express in heat-stressed seedlings, A2, A3, A6, A7, B2 and B5 were exclusively heat induced and other HSF subclasses could also be involved in other abiotic stress responses (Lin et al., 2014). qPCR expression analysis of chickpea HSFs under HS at pod development and at 15 days old seedling stage showed that CarHSFA2, A6, and B2 were constitutively up-regulated at both plant development stages indicating their importance in the regulatory network relative to HS (Chidambaranathan et al., 2018). In the present study, various transcripts of putative pea HSFs were characterized that were responsive to 3 h heat treatment, among which putative HSFA stood out in its amount abundance, the A3 subclass in particular. Three HSFA transcripts (Psat3g061600, Psat5g036400 and Psat6g059040) were highlighted because their transcriptional levels were dominantly increased in both anthers and stipules in all three varieties (Table 4.4), suggesting they are essential transcriptional regulators in pea HSR. A further analysis on knock-out mutants of these HSF genes will validate their exact roles, whether directly or not, in heat regulation. Interesting, individual HSF were identified for anthers and stipules, indicating different regulatory networks may exist between vegetative and reproductive organs.

Functions and molecular mechanism of HSFBs were less elucidated, but they were found to interact closely with HSFA in plant's HSR. The role of HSFBs were reported either as a repressor or an activator in the transcription of HSFA depending on plant species, as a result, they participated in different mechanisms in HS regulation. In *A. thaliana*, HSFB suppressed the transcriptional activities of HS-inducible HSFs, including HSFA2, A7a, at both normal temperature environment and HS condition (Ikeda et al., 2011). On the contrary, the function of tomato's HSFB1 seemed more complex, it could work either as a co-activator of some HSFs e.g., HSFA1a or as a transcription repressor of other HSFs such as HSFA1b and HSFA2 (Bharti et al., 2004; Fragkostefanakis et al., 2019; Hahn et al., 2011). In our result, transcription levels of two putative HSFB2 genes (Psat1g102600 and Psat6g200480) were highly heat induced along with HSFA genes independent of organ types and genotypes, implying their positive role in the transcriptional regulation of field pea in HS, which was in agreement with the finding in chickpea (Chidambarabathan et al., 2018). It seemed that the role of HSFB in legume crops was similar to the coactivator characteristics of tomato HSFB.

4.5.3 Transcriptional induction of various pea small heat shock proteins and heat shock protein 70s at heat stress

In plant cellular defense against HS, the induction of HSP is one of the major responses. HSPs act as molecular chaperones which are proteins that facilitate folding of other functional proteins especially at the secondary and tertiary structure and prevent them from denaturation and aggregation during exposure to HS. Depending on the molecular size, HSPs are divided into five conserved classes: sHSPs, HSP60, HSP70, HSP90 and HSP100.

Small heat shock proteins range in size from 10 to 42 kDa and share a conserved C-terminal domain that is common to all eukaryotic organisms. Generally, sHSP functions as a molecular chaperone and protects the substrate proteins against thermal aggregation or denaturation. In six legume species, more than 5 different sHSPs were detected from plant tissues exposed to HS (Hernandez and Vierling, 1993). In pea, several sHSPs belonging to two classes based on their sequence alignment and immunological cross-reactivity were isolated. *PsHSP 17.7, 17.9, 18.1* were located in the cytoplasm, whereas *PsHSP21 and PsHSP22* were located in chloroplasts and mitochondria, respectively (DeRocher and Vierling, 1995; DeRocher et al., 1991; Lenne et al., 1995). From these reports, we could conclude that they were all involved in establishing cellular thermotolerance to some degree, though the induction of their expression was triggered at different temperatures. Transcriptome profiling in our experiment revealed that the transcriptional levels of cytoplasmic sHSPs were drastically increased at HS among the three pea varieties (Table 4.4), which was in agreement with the above-mentioned result on other pea genotypes, suggesting the function of these sHSPs is general in field pea plant. Beyond that, transcriptional response of other sHSPs in relation to HS were also characterized, which provides a more comprehensive picture of sHSP relating pea HSR.

Heat shock protein 70s have also been extensively studied; they are ATP-driven molecular chaperones with an N-terminal ATPase domain and a C-terminal peptide binding domain. Similar to the gene family encoding sHSPs, HSP70 genes also encode proteins targeted to different cellular compartments, including mitochondria, chloroplast, endoplasmic reticulum, and the cytoplasm. Similarly, HSPs isolated in pea differed in their expression under different temperature environments, inferring functional differences between heat-induced and

constitutively expressed HSP70 homologues. In our study we confirmed the significance of various HSP70 genes in field pea HSR.

4.5.4 Heat stress response in pea cell wall

MapMan analysis of HS related metabolic changes indicated that various cell wall related pathways were highly responsive in both anthers and stipules of pea varieties (Fig 4.7, 4.8). And GO enrichment analysis further revealed that various biological processes relating to cell wall were significantly down-regulated when exposed to HS in our study, which helped decipher the molecular mechanism of heat damage on pea cell wall (Fig 4.5 and 4.6). Similar in heat stressed lentil, a major group of heat responsive genes were involved in plasma membrane and cell wall (Singh et al., 2019).

Plant cell wall has multiple layers and is made up of three sections, i.e., the middle lamella, primary cell wall, and secondary cell wall. The primary wall surrounds growing cells or cells capable of cell growth and its plasticity is essential for cell expansion and growth; whereas the secondary wall is a highly specialized and thickened structure to provide the sufficient rigidity, which undergoes irreversible changes in many fully developed cells. The middle lamella is a pectin layer to provide necessary adhesive between two adjoining cells (Wu et al., 2018). Pectin, a mixture of polysaccharides, is also a major composition in primary cell wall, especially in dicotyledonous plants (Mohnen, 2008). In addition to its adhesive property, an adjustment of its content in cell wall is proposed to link with various physiological functions during plant life cycle as well as contribute to signal transduction to various conditions. Reproductive tissues are particularly rich in pectin compared with other tissues, for example pectin constituted ~40% and 15% in rice pistil and anther cell wall, respectively, whereas the proportion of pectin was only 5% in the cell wall of mature leaf (Hasegawa et al., 2020). Transcriptome comparison of this study between HS and normal temperature characterized a cluster of genes encoding pectin esterase (enzymes for pectin metabolism), only heat responsive in anthers of all three varieties, not in stipule, and it is proposed to be associated with the contrasting pectin composition between reproductive organ and vegetative plant organ. The reduced expression of pectin methyl esterase (PME; EC 3.1.1.11) genes under HS was consistent with the finding in canola (Yu et al., 2014). Intriguingly, recent studies in pea aluminum stress and cold stress suggested that the degree of pectin methyl-esterification and PME activity could also play a role in both abiotic

stresses (Baldwin et al., 2014; Li et al., 2016). Still, the stress effect on the architecture of cell wall remodeling by PME activity may depend on the plant species, genotype, and growth stage, and also rely on the intensity and timing of the stress (Wu et al., 2018).

Lignin is a major composition in secondary cell wall and provides cell structural rigidity. Its biosynthesis consists of a very complicated network, where cinnamyl alcohol dehydrogenase (CAD), laccase (LAC) and peroxidase are involved. In *A. thaliana*, CAD function defective mutant displayed inhibited plant and male sterile compared with wild type, likely attributed to the abnormally reduced lignin biosynthesis in the anther (Thévenin et al., 2011). Likewise, CAD1 mutant of *Medicago truncatula* had a much lower lignin content than the wild type, though causing no growth difference between two materials at normal temperature environment (22°C), the growth of this *MtCAD1* mutant was significantly suppressed at 30°C (Zhao et al., 2013). In our study, lignin metabolic and catabolic process was identified to be uniquely up-regulated in the anther's transcriptome of heat tolerant variety, PR11-2, when exposed to HS (Fig 4.6). The genes in this process were identified to be LAC encoding genes on pea chromosome II, III, V and VII, which are predicted to be associated with heat tolerance. In Anadiplosis, functions of LAC 1, 4 and 17 were linked with anther dehiscence success (Zhao et al., 2013). A QTL was identified for HS susceptibility index of percent spikelet sterility in rice on chromosome XII, and one LAC gene was included in this QTL interval (Shanmugavadivel et al., 2017).

4.5.5 Effects of heat stress on lipid transport and metabolism

Heat stress in this study adversely affected lipid transport and localization in both pea anther and stipule independent of genotypes (Fig 4.5). The lipid process was inhibited mainly via the downregulation of various transcripts encoding non-specific lipid transfer proteins (LTPs; Table 4.5). Plant LTPs are broadly categorized into LTP1 and LTP2 groups based on the molecular weighs. LTP1s generally consist of 90 amino acids, whereas LTP2s have around 70 amino acids. Although the biological functions of LTPs have not been clear yet, previous studies suggested that LTPs genes can be divided into three groups depending on expression patterns of the related genes, that is, 1) genes only expressed in aerial plant parts; 2) genes only expressed in root; and 3) genes whose expression was restricted in reproductive tissues (Salminen et al., 2016). Our results added another piece of evidence to support the tissue-specific expression of LTP genes, because different transcripts of LTP genes were characterized between field pea

anther and stipule at normal development as well as at HS condition. Except that the two genes encoding *PsLTP1* and *PsLTP2*, previously isolated in pea seeds (Bogdanov et al., 2016), were heat responsive in both plant samples, other corresponding genes variated. To the authors' knowledge, our work is the first to report the link between LTP genes with pea normal plant development and HSR, and their biological functions are worth being validated via mutation experiment. In wheat, *LTP3* accumulation was detected in cell membranes after HS at plant seedling and grain-filling stages; transgenic Arabidopsis seedlings overexpressing *TaLTP3* were more tolerant to HS than control plants, possibly because of less membrane injury (Wang et al., 2014).

In addition, the lipid metabolic process was damaged by HS in both anther and stipule among all three pea varieties (Figs 4.5 and 4.7), which was also seen in rice heat stressed anther (Endo et al., 2009). The damage was mostly due to that the transcriptional activity of multiple genes associated with GDSL lipase were adversely affected, although GDSL gene family was differentially expressed between anther and stipule (Table 4.5). Studies in this aspect are scarce in legume including pea. In the model plant *A. thaliana*, GDSL lipase gene has a family of 108 gene members, which are distributed across plant genome (Ling, 2008; Lai et al., 2017). Among them, 20 members were expressed in all tissues, and the other 16 and five members were exclusively expressed in flower and root, respectively. One GDSL lipase was reported to be involved in the formation of pollen coat (Mayfield et al., 2001). With the advance in omics technology, the integration of lipidome and transcriptome provides a new perspective of studying HS as shown in Higashi et al. (2015).

4.5.6 Coincidence of heat responsive genes among field pea studies

Genomic understanding of pea HS and selection for heat tolerant varieties started in the past decade, benefiting from the rapid advancement in sequencing technology. However, results from individual research projects cannot be sufficiently compared because the types of genetic markers applied differed. Our characterized heat responsive genes can be compared with a recent association mapping study by Tafesse et al. (2020), as pea genome locus markers were used in their work. Twelve DEGs in this study coincided with putative candidate genes for heat responsive trait characterized in the field condition from their work (Appendix E). The response of these 12 genes fell into three patterns: 1) responsive in all tissue types among the three

varieties (e.g., Psat5g303760 encoding uncharacterized protein); 2) specifically responsive to tissue type (e.g., Psat2g144160 encoding pectin acetylesterase); 3) only responsive in certain genotype (e.g., Psat2g166520 encoding putative rapid alkalinization factor). Further functional annotation of individual gene would benefit to explicit its role in HSR.

4.6 Conclusions

This chapter profiles a global transcriptome response to short term HS among different field pea varieties. A full set of heat responsive genes are characterized, including HSP genes reported in the previous literature. Common effects of HS in biological processes are shared between the anthers (reproductive organ) and the stipules on the same flowering node (vegetative organ), though the involved genes in certain processes differed between the two organs (e.g., lipid transport and metabolic process). Distinct heat responses are characterized on individual pea varieties, which provides insight into the molecular mechanisms of heat-tolerance response. Overall, this research supports the utilization of RNA-Seq for the identification of heat inducible genes, and addresses the three core purposes proposed in the chapter introduction, which are 1) the characterization of additional gene response toward high temperature besides previously characterized pea HSP genes; 2) comparative analyses of heat responsive gene expression differences between anthers, representative of the reproductive plant parts, and stipules, representative of the vegetative plant parts; 3) comparisons between heat tolerant and heat susceptible varieties for an enhanced understanding of pea heat tolerance and susceptibility from the view of gene response. The variety's heat responsive genes provide a preliminary gene list for marker design to select pea heat tolerance.

Transition section between Chapter 4 and Chapter 5

In chapter 4, variety specific heat responsive genes were successfully characterized among PR11-2, PR11-90 and CDC Amarillo. A population of 39 RILs from the cross of PR11-2 and CDC Amarillo was used as plant materials in the following chapter, and this population was tested for heat responsive traits in field trials. By genotyping the population in parallel, genetic mapping of these traits was conducted. The corresponding genome loci identified will serve as validation of the findings in chapter 4. The heat stress traits measured were a subset of my previously published manuscript on PR-11 (Huang et al., 2017). The results of chapter 5 can verify the QTLs reported from PR-11 and other pea populations in a different genetic background. At the same time, novel loci can also be found as the measured traits are believed to be controlled by multiple genes. In addition, other agronomic traits including lodging resistance and seed composition were characterized in this population.

CHAPTER 5. CHARACTERIZATION OF QTLs RELATED TO FLOWERING, YIELD COMPONENTS AND OTHER AGRONOMIC TRAITS UNDER NORMAL AND HEAT STRESS ENVIRONMENTS IN A PEA RECONBINANT INBRED LINE POPULATION

This chapter is targeted for submission to Canadian Journal of Plant Science in July or August 2022 for publication.

'**Huang, S**., Gali, K.K., Arganosa, G.C., Bueckert, R.A., Tar'an, B., and Warkentin, T.D. Breeding indicators for high-yielding field pea under normal and heat stress environments.'

5.1 Abstract

The warming Canadian summers have become a major abiotic stress to crops including pea. In the past decade, attempts were made in the understanding of heat stress (HS) effects and genomic mapping for heat responsive traits in field pea. In this study, a new recombinant inbred line population consisting of 39 lines was tested in six trials in the summers of 2020 and 2021. Data related to days to flowering (DTF), days to maturity (DTM), plant height, lodging, yield components, plot yield and seed quality were collected in individual trials. Plant height could be an effective indicator for yield prediction, because its correlation with plot yield was significantly positive in all six trials with varying degrees of heat and drought stresses. Correlation analyses suggested desirable traits varied under different temperature environments, that i.e., under normal summer climate, a relatively late maturity could contribute to a better yield potential; under HS environment, yield success on the mainstem was important. Linkage mapping was used to dissect the genomic regions associated with the measured traits. Four QTLs were characterized over multiple trials, one each for DTF (chromosome 7), DTM (chromosome 5), reproductive node number (RNN; chromosome 5), and PN (chromosome 2). These loci are worth fine-mapping for putative gene discovery.

5.2 Introduction

Heat stress refers to the detrimental effects of temperatures beyond the upper-temperature threshold of a plant's normal growth and development. In western Canada, the threshold maximum temperature for pea yield reduction in the field was approximately 28°C daily maximum at reproductive stage, and above 17.5°C mean seasonal daily temperature (Bueckert et al., 2015). High ambient temperature at flowering stage shortened pea′s flowering duration and caused young pod abortion (Huang et al., 2017). Traits relating to flowering, yield and heat tolerance were confirmed as quantitative traits which were controlled by multi-gene action (Huang et al., 2017; Jiang et al., 2017; Tafesse et al., 2020). To gain knowledge on the quantitative traits genetically, a useful method is to build a genetic linkage map, and thereby characterize corresponding quantitative trait loci via the integration of phenotypic data. Huang et al. (2017) identified ten consistent QTLs associated with flowering and yield component traits, in particular, a QTL for DTF on chromosome 1 consistent across four field trials varying in temperature stresses. The QTLs for flowering duration, thousand seed weight and RNN were different between normal and late seeding, which implies different mechanisms were involved under contrasting temperature environments. However, different genome loci were identified for the same traits in different genetic backgrounds (Jiang et al., 2017; Tafesse et al., 2020).

In this chapter, PR-24 was used as a new pea recombinant inbred line (RIL) population for QTL analysis of flowering, yield traits, other agronomic traits, as well as seed composition. It is hypothesized that some of the identified QTLs would be at similar genome loci reported in other pea populations, especially in Canadian pea populations, for marker validation purpose, and novel QTLs would be characterized in this new population.

5.3 Materials and Methods

5.3.1 PR-24 population development

A population of 39 RILs, namely PR-24, was developed from the cross of PR11-2 and CDC Amarillo by Dr. Gali at the Crop Development Centre, University of Saskatchewan. Each line was derived from a single F2 seed, and the generations were advanced to F7 by single seed descent in the Agriculture Greenhouse, University of Saskatchewan. PR11-2 is a green pea variety developed from the cross of yellow pea cultivar CDC Centennial (Warkentin et al., 2007) and green pea cultivar CDC Sage (Warkentin et al., 2006). It is considered as a heat tolerant

variety due to consistently high yield potential under normal and heat stress field environments (Huang et al., 2021). CDC Amarillo is a yellow pea variety and one of the best yielding varieties in western Canada (Warkentin et al., 2014). The descriptions of PR11-2 and CDC Amarillo were fully covered in '4.3.1 Plant Material'.

5.3.2 Field trials

The field experiment started in 2020 at Saskatoon (52º12'N, 106º63'W; Dark Brown Chernozem), Rosthern (52º66'N, 106º33'W; Orthic Black Chernozem) and Lucky Lake (50º98'N, 107º13'W; Dark Brown Chernozem) in Saskatchewan, Canada. Within each site, a randomized complete block design with three replications was used. Eighty four seeds of individual RILs were planted in 1 m × 1 m micro-plots with three rows with 30 cm row spacing. The field trial was managed via best management practices for pea in western Canada. Due to unexpected early season sandblasting at Saskatoon, plants were heavily damaged and discarded for analysis. The field trial was repeated in 2021 at Saskatoon and Rosthern in Saskatchewan, Canada. At each location, a normal seeding date and late seeding date trial with three replicates were planted. The late seeding date was expected to shift the flowering period to a higher temperature environment in late July and early August, and is generally considered as a more heat stressful environment. Collectively, six sets of field data were produced over two years' field experiments, namely, 2020 Rosthern (2020_ROS), 2020 Lucky Lake (2020_LL), 2021 Saskatoon early (2021_SAS_E), 2021 Saskatoon late (2021_SAS_L), 2021 Rosthern early (2021_ROS_E), and 2021 Rosthern late (2021_ROS_L).

5.3.3 Phenotyping

Starting from mid-June, the field trials were visited on a weekly basis. DTF was noted when half of the plants in a microplot started to flower. When plant development was near physiological maturity, three single plants with average height were randomly picked within individual micro-plots, whose RNN and PN on the main stem were manually counted. DTM was noted for each RIL when 80% of the pods in a microplot had turned brown. In addition, the grain yield of each RIL micro-plot was measured after combine harvest.

Proximate composition was predicted by a FOSS NIR System Model 6500 near infrared spectrophotometer using in-house equations for protein, starch, acid-detergent fiber (ADF), neutral detergent fiber (NDF), ether extract (EE), and ash. Moisture was tested on a subset of

samples (AACC Method 44.17.01) and all data were reported on a dry matter basis (Arganosa et al., 2006). The lab assay was conducted by Dr. Arganosa and Mrs. Weerasinghe at the Grains Innovation Lab, Crop Development Centre, University of Saskatchewan.

5.3.4 Heat tolerance indices calculation

Based on their reported effectiveness for predicting crop tolerance under abiotic stress in previous literature, five abiotic stress indices were evaluated in this study, and their calculation formulas are listed below.

Stress Susceptibility Index (SSI) = $(1 - Y_{stress}/Y_{control})/ (1 - \bar{Y}_{stress}/\bar{Y}_{control})$, where $\bar{Y}_{stress}$ and $\bar{Y}_{control}$ were the mean yield of all RILs in stressed and control environments, respectively (Fisher and Maurer, 1978).

Geometric Mean Productivity (GMP) = $\sqrt{(Y_{control} \times Y_{stress})}$ (Fernandez, 1992)

Stress Tolerance Index (STI) = $(Y_{control} \times Y_{stress})/(\bar{Y}_{control})^2$ (Fernandez, 1992)

Yield Stability Index (YSI) = $Y_{stress}/Y_{control}$ (Bouslama and Schapaugh, 1984)

Abiotic Tolerance Index (ATI) = $(Y_{control} - Y_{stress})/(\bar{Y}_{control}/\bar{Y}_{stress})$ (Mousavi et al., 2008)

5.3.5 Genotyping

Genotyping was conducted with the guidance of Dr. Gali at the Pulse Crop Molecular Breeding Lab, University of Saskatchewan. DNA of individual RILs and the two parents were extracted using DNeasy Plant Mini Kit (Qiagen, Germany) and DNA concentration was quantified at optical density 260 nm in Nanodrop 8000 UV spectrophotometer. Then DNA stock was diluted to 10 ng μl^{-1} standard concentration for KASP array genotyping (LGC, UK).

One hundred and seventy seven genome-wide KASP markers were tested for polymorphism between the two parental lines, and 88 markers were shown putatively polymorphic. These 88 markers were subsequently used to genotype the 39 RILs. KASP assays were prepared in 384-well plates, and in a single well, 3 µl DNA of individual RIL was mixed with 3 µl KASP master mix, as well as 0.084 ul assay mix consisting of a combination of allele-specific primers and common reverse primer. KASP genotyping was conducted using BIO-RAD CFX384 instrument and the PCR conditions were according to suggested protocol. For each locus, RILs were assigned to either the allele of CDC Amarillo, the allele of PR11-2, or heterozygote based on their grouping into the respective clusters.

965 5.3.6 Statistical analysis

966 Analysis of variance was conducted for individual traits of interest via PROC MIXED
967 program (SAS version 9.4), where genotypes, location and genotype-by-location were
968 considered as fixed effects.

969 After genotyping on 88 SNP markers, 64 polymorphic markers were selected for
970 construction of a linkage map, based on the quality of PCR amplification and separation between
971 parental lines and among RILs, and were used to make the genetic map. For the linkage map
972 construction, these polymorphic SNP markers were clustered into different linkage groups based
973 on a minimum LOD (logarithm of odds ratio) value of 5 using QTL IciMapping (Meng et al.,
974 2015). Then the map order of each linkage group was finalized with the use of regression
975 mapping. The recombination frequencies were converted into centiMorgan (cM) through the
976 Kosambi mapping function. A graphical map was generated by QTL IciMapping (Meng et al.,
977 2015).

978 For individual traits, QTL analysis was conducted on the phenotypic data of separate
979 locations via inclusive compositive interval mapping program in QTL IciMapping. The QTL
980 were filtered to select those where the LOD score was above the threshold value 2.0.

981 **5.4 Results**

982 5.4.1 Weather summary of field locations in 2020 and 2021 summer

983 Summer weather data of 2020 and 2021 Saskatoon and Lucky Lake were separately
984 retrieved from the meteorological station Saskatoon Rcs and Lucky Lake from Environment
985 Canada historical weather database. For Rosthern, its historical weather data were retrieved from
986 Worldweatheronline at the following link, https://www.worldweatheronline.com/rosthern-
987 weather-history/saskatchewan/ca.aspx. In 2020, PR-24 population experienced similar HS level
988 at two locations, and the HS level was also similar to the previous years' pea trials in 2013-2017,
989 whose environment conditions were grouped as control HS environment (Huang et al., 2017;
990 Tafesse et al., 2020). Therefore, data arising from 2020 Lucky Lake and 2020 Rosthern were
991 considered as 'control conditions' in this study. In 2021, pea plots experienced a greater number
992 of extremely hot days at both vegetative and reproductive stages than pea plots in 2020, thus all
993 four trials in 2021were grouped as 'HS condition' (Table 5.1). Along with the hot temperature

994 and drying wind throughout the summer in 2021, precipitation was also limited, especially rainfall at the reproductive stage.
995 Insufficient topsoil moisture and high temperature resulted in lower pea yield in 2021 than 2020.

996 Table 5.1. Seeding dates, average population flowering dates, number of days with daily maximum temperature above 28°C and
997 cumulative precipitation at vegetative and reproductive stages.

Trials	seeding dates	First flowering date	number of days max Temp >28C VS	number of days max Temp >28C RS	total rainfall (mm) VS	total rainfall (mm) RS	average population yield (kg/ha)	stress group
2020_LL	May 22	July 10	3	10	101	51	1034	control
2020_ROS	May 25	July 14	3	11	207	33	1420	control
2021_SAS_E	May 5	July 1	11	23	77	18	892	hot, dry
2021_SAS_L	May 28	July 12	17	20	49	18	953	hot, dry
2021_ROS_E	May 11	July 1	11	19	115	9	365	hot, dry
2021_ROS_L	May 31	July 10	16	19	68	9	233	hot, dry

998 Note: LL, Lucky Lake; ROS, Rosthern; SAS, Saskatoon; E, early seeding; L, late seeding; Temp, temperature; mm, millimeter; VS,
999 vegetative stage from seeding to late vegetative stage; RS, reproductive stage from beginning of flowering to physiological maturity.
1000 Weather data of LL and SAS were sourced from Environment Canada, and weather data of Rosthern were sourced from
1001 worldweatheronline.

1002

5.4.2 Combined analysis of variance for flowering and yield component traits

To assess the environmental and genotypic effects on phenotypic traits relating to flowering, yield components and agronomy, a combined analysis of variance with six environments, 39 RILs and their interaction as fixed effects was conducted on individual traits. DTF, plant height, lodging, DTM, RNN, PN and yield all varied depending on field environment (Table 5.2). All traits except for lodging were also significantly affected by genotypic variability within PR-24 population. In addition, significant interactive effect between environment and genotype was observed in traits of DTF, DTM, RNN and PN.

Table 5.2. Analysis of variance with F value for days to flowering, days to maturity, reproductive node number, pod number, and plot yield of PR-24 population among six field trials at Saskatoon, Rosthern and Lucky Lake over 2020-2021 summers.

Factor	DF	DTF	height	lodging	DTM	RNN	PN	yield
E	5	1562.6***	536.7***	23.3***	1381.2***	1538.3***	1604.2***	631.2***
G	38	12.4***	1.89**	1.2ns	5.9***	4.2***	4.8***	2.4*
G x E	190	1.44**	1.12ns	1.1ns	1.3*	4.2***	4.6***	1.1ns

Notes: E, environment (six field trials); G, genotype; G x E, genotype by environment interaction; DF, degree of freedom; DTF, days to flowering; DTM, days to maturity; RNN, reproductive node number; PN, pod number; yield, plot seed yield in kg ha^{-1}. ns, not significant; *, significant at 0.05 probability level; **, significant at probability of 0.01; ***, significant at probability of 0.001.

5.4.3 Combined analysis of variance on seed quality traits

All six seed component traits were significantly affected by environmental variation over two years (Table 5.3), implying pea seed quality could be highly dependent on growth location and climatic change. Significant genotypic variation of seed composition in PR-24 indicated the potential to select RIL with better seed quality within this genetic population.

Table 5.3. Analysis of variance with F value for seed composition of PR-24 population at six field trials in 2020 and 2021 summer.

Factor	DF	protein	starch	NDF	ADF	EE	ash
E	5	126.1***	111.4***	78.1***	36.7***	184.6***	494.0***
G	38	2.4***	5.5***	12.9***	3.8***	10.6***	6.3***
G x E	190	1.1ns	2.0***	2.9***	1.5***	2.1***	1.7***

Notes: NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ether extract. Factor names and significant descriptions refer to Table 5.2.

5.4.4 Correlation analysis between plot yield and traits of days to flowering, days to maturity, lodging, plant height, reproductive node number and pod number in individual field trials

With a primary focus on yield performance, it was interesting to find the relationship of plot yield with other measured traits among the 39 RILs in the population. Because most of phenotypic traits were significantly affected by environment and GXE interactions (Table 5.2), correlations between yield and other traits were analyzed for individual trials. Plant height had a significantly positive correlation with plot yield in all six trials (Table 5.4). Lodging had negative correlation with plot yield in four out of six trials, suggesting that varieties with less lodging would have promising yield potential regardless of climatic summer conditions in Saskatchewan. Positive correlation between DTF and yield was significant at control environment (2020_LL and 2020_ROS), and HS environment with less drought (2021_SAS_E and 2021_SAS_L); but the correlation became significantly negative at 2021_ROS_E and 2021_ROS_L, where heat and drought were both extremely stressful in this year. Under control environments, favorable for pea growth, RILs with relatively late maturity were likely to yield more, which were also reflected by the significantly positive correlation between DTM and yield at 2020_LL and 2020_ROS. Under the four stressful trials, DTM was positively correlated with yield at 2021 SAS_E, and the correlations were not statistically significant at the other trials. PN was also positively correlated with yield in four of the six trials, and it could be an effective indicator for yield prediction in most conditions.

Table 5.4. Correlation coefficients between plot yield and other measured traits at individual summer trials.

trial name	DTF	height	lodging	DTM	RNN	PN
2020_LL	0.40***	0.51***	-0.41***	0.29**	0.13ns	0.20*
2020_ROS	0.27**	0.52***	-0.37***	0.60***	0.13ns	0.16ns
2021_SAS_E	0.24*	0.35***	-0.11ns	0.22*	0.19*	0.16ns
2021_SAS_L	0.18*	0.55***	0.01ns	0.18ns	0.03ns	0.21*
2021_ROS_E	-0.21*	0.65***	-0.22*	0.06ns	0.42***	0.54***
2021_ROS_L	-0.33***	0.69***	-0.34***	0.02ns	0.70***	0.72***

Note: full trial names refer to Table 5.1, and the descriptions of trait names and statistical significance refer to Table 5.2; statistical significance was based on N = 39 (RIL number) X 3 (replicate number at individual trial).

5.4.5 Quantitative trait loci analysis

To dissect the genomic regions associated with the measured phenotypic traits, QTL analysis was conducted based on the association of phenotypic data with genotypic data. Firstly, each RIL was genotyped by 88 KASP markers that were tested polymorphic between the two parental varieties, PR11-2 and CDC Amarillo, where 64 KASP markers were eventually polymorphic among RILs. Sixty out of these 64 KASP markers were successfully clustered into seven pea linkage groups (LGs) and used to construct the PR-24 genetic map (Table 5.5). Although marker density is low, common markers to the pea consensus linkage map of Sindhu et al. (2014) were successfully aligned, confirming the quality and accuracy of the PR-24 linkage map built. In the PR-24 linkage map, markers in both LG VI and VII were aligned to the markers of LG VI in the pea consensus map, however due to the lack of anchor markers, these two LGs were assigned as separate groups. For the same reason, LG VIII and IX in this map both aligned with LG VII of pea consensus map but were assigned into separate groups.

Table 5.5. PR-24 genetic map and its alignment with pea reference genome (Kreplak et al., 2019) and the earlier pea consensus map (Sindhu et al., 2014).

Name of marker	Linkage group	Position (cM)	SNP position on pea reference genome[a]	Linkage group clustered in the earlier pea consensus map[b]
PsC25617p184	I	0	Chr2LG1_8794844	I
PsC20526p286	I	30.1	Chr2LG1_19046389	I
PsC26900p157	I	30.1	Chr2LG1_29676358	I
PsC14530p126	I	35.3	Chr2LG1_13125801	I
PsC13109p598	I	35.3	Chr2LG1_13138279	I
PsC25275p166	I	64.1	Chr2LG1_375508603	I
PsC2159p286	I	71.7	Chr2LG1_386850165	I
PsC6329p79	I	113.4	Chr2LG1_314673840	I
PsC5029p497	I	116.8	Chr2LG1_302559476	I
PsC6221p181	I	120.3	Chr2LG1_386249244	I
PsC24631p193	I	166.4	Chr2LG1_425414554	I
PsC2169p461	II	0	Sc01315_15204	II
PsC6145p417	II	82.4	Chr6LG2_25590723	II
PsC25874p265	III	0	Chr5LG3_136123241	III
V-7	III	3	Chr5LG3_217884076	Unknown
V-1	III	7.6	Chr5LG3_217458441	Unknown
PsC5352p274	III	27.7	Sc03550_21667	III
PsC4623p176	III	59	Chr5LG3_234878784	III
PsC13141p220	III	63.7	Chr5LG3_287795726	III
PsC5404p543	III	83.4	Chr5LG3_490393084	III
PsC2870p236	III	94.7	Chr5LG3_415679416	III
PsC3195p368	III	119.2	Chr5LG3_198234688	III
V-9	III	136.5	Chr5LG3_572899434	Unknown
PsC4281p586	III	141.1	Chr5LG3_130378814	III
PsC14297p386	IV	0	Chr4LG4_101151915	IV
PsC28982p67	IV	40.2	Chr4LG4_25656691	IV
PsC5966p69	IV	82.4	Chr4LG4_326445536	IV
PsC3577p93	IV	95.2	Chr4LG4_28425329	IV
PsC20442p333	V	0	Chr3LG5_215356668	V
V-12	V	21.2	Chr3LG5_98748132	Unknown
V-11	V	58.4	Chr3LG5_126657675	Unknown
PsC26393p87	V	87.3	Chr3LG5_418943258	V
PsC5316p234	V	134.5	Sc01923_32192	V
PsC4403p76	V	170	Chr3LG5_421825065	V
PsC12889p283	V	171.7	Chr3LG5_424842067	V
PsC14430p66	VIa	0	Chr1LG6_280392484	VI
PsC3267p192	VIa	11.6	Chr1LG6_76570979	VI
PsC4675p73	VIa	18.5	Chr1LG6_314147029	VI
PsC26551p132	VIa	41.1	Chr1LG6_87293371	VI

PsC13805p217	VIa	76.4	Chr1LG6_370510470	VI
PsC4052p342	VIa	81	Chr1LG6_365300963	VI
PsC1405p153	VIa	85.4	Chr1LG6_369854314	VI
PsC13253p612	VIa	110.2	Chr1LG6_321500925	VI
PsC28635p290	VIa	111.7	Chr1LG6_325742168	VI
PsC3875p766	VIa	131.8	Chr1LG6_264505999	VI
PsC22752p338	VIa	177.4	Chr1LG6_76697841	VI
PsC6423p69	VIb	0	Chr1LG6_316735094	VI
PsC27596p426	VIb	53.5	Chr1LG6_133794532	VI
PsC4963p219	VIIa	0	Chr7LG7_59679004	VII
PsC4676p597	VIIa	50.4	Chr7LG7_187701067	VII
PsC27413p94	VIIb	0	Chr7LG7_15445120	VII
PsC21060p179	VIIb	4	Chr7LG7_28454801	VII
PsC14648p140	VIIb	24.5	Chr7LG7_151291468	VII
PsC13180p419	VIIb	30.6	Chr7LG7_126083998	VII
PsC4635p485	VIIb	37.5	Chr7LG7_169190602	VII
PsC4056p224	VIIb	49.5	Chr7LG7_165950533	VII
PsC6058p226	VIIb	64.2	Chr7LG7_67444481	VII
PsC27183p403	VIIb	93.1	Chr7LG7_488920599	VII
PsC2575p276	VIIb	105	Chr7LG7_78466126	VII
PsC3005p202	VIIb	136.3	Chr7LG7_26298790	VII

[a] SNP marker physical location on pea genome was aligned with pea reference genome (Kreplak et al. 2019).

[b] Assigned linkage group of individual markers in the earlier pea consensus map (Sindhu et al. 2014).

Based on the linkage map and phenotypic data, QTL analysis of individual traits was conducted on each location to dissect the underlying genetic mechanism in trait development. Particular attention was paid to flowering, yield components and plot yield, as these traits were more heat susceptible and closely linked with heat tolerance. Various QTLs associated with individual traits were found among different field experimental environments, which could be partially reflected by the significant environmental and G by E effects in many traits (Table 5.2).

For DTF, different QTLs were identified between control and HS environment groups, suggesting different genetic mechanisms were involved in the control of flowering time at contrasting temperature conditions. No common QTL was found between two control environments, 2020_LL and 2020_ROS, but a stable QTL for DTF at HS environment was found on pea LG VII, corresponding with pea chromosome 7 (Table 5.6). This QTL was detected at three out of four heat stressful trials except 2021_SAS_E, and it was also found at

2020_LL. Four markers were within this QTL in the genetic map, which were between 6744481 and 169190602 base pair (bp) on pea chromosome 7. Among the six QTLs for DTM, two QTLs overlapped at LG III and shared the same right flanking marker 'PsC5404p543', which was positioned at 490393084 on pea chromosome 5. One of the two QTLs was detected at 2021 Saskatoon early seeding trial and the other QTL was found at 2021 Saskatoon late seeding trial, with each accounting for ~20% of the phenotypic variation. For lodging, height and plot yield, no consistent QTL was detected over multiple trials for these traits.

Likewise different QTLs for PN were characterized between control and HS environment groups. A QTL at LG I was considered most potentially linked with genetic control of pod number at HS, as it was identified in more than one trial of the four at HS environment group. The left flanking marker was at nucleotide position 314673840 on chromosome 2 and the other flanking marker was at nucleotide position 386850165 on the same chromosome. Similarly, a common QTL associated with RNN was identified at LG III in multiple trials, which corresponded with the physical interval on chromosome 5 from nucleotide position 198235688 to 415679416. Another QTL at LG VI was interesting and seemed to link with PN and RNN at 2021 Rosthern early seeding trial. It accounted for 16% of the phenotypic variation of RNN and 22% of the variation of PN at 2021_ROS_E. The genes within these stable QTLs detected over multiple trials would be further evaluated, and their possible relationship with pea heat tolerance would be discussed by comparing with the findings in the previous thesis chapter of heat responsive gene discovery, as well as previous QTL findings in other manuscripts.

Table 5.6. Summary of identified quantitative trait loci for days to flowering, lodging, days to maturity, pod number in PR-24 pea population at Saskatoon, Rosthern and Lucky Lake in 2020 and 2021.

QTL symbols	Traits	trials	LG	peak position	LOD	PVE	Left flanking marker	Right flanking marker
DTF-1	DTF	2021_SAS_E	7b	60	2.3	27.1	PsC4056p224	PsC6058p226
DTF-1	DTF	2021_ROS_E	7b	43	2.8	25.2	PsC4635p485	PsC4056p224
DTF-1	DTF	2021_ROS_L	7b	38	3	29.7	PsC13180p419	PsC4056p224
DTF-1	DTF	2020_LL	7b	37	2.3	23.9	PsC13180p419	PsC4056p224
DTF-2	DTF	2020_LL	6a	110	2.0	21.5	PsC1405p153	PsC28635p290
DTF-3	DTF	2020_ROS	5	81	2.2	22.5	V-11	PsC26393p87
DTF-4	DTF	2021_SAS_E	1	72	2.2	17.7	PsC2159p286	PsC6329p79
PN-1	PN	2021_SAS_E	1	98	2	15.3	PsC2159p286	PsC6329p79
PN-1	PN	2021_ROS_L	1	73	2.8	27.7	PsC2159p286	PsC6329p79
PN-2	PN	2020_LL	3	39	2.3	24.6	PsC5352p274	PsC4623p176
PN-3	PN	2020_ROS	7b	3	2.2	22.7	PsC27413p94	PsC21060p179
PN-4	PN	2021_SAS_E	4	61	2.7	17.4	PsC28982p67	PsC5966p69
PN-5	PN	2021_ROS_E	6b	18	2.1	21.8	PsC6423p69	PsC27596p426
RNN-1	RNN	2021_SAS_L	3	106	2	23.2	PsC2870p236	PsC3195p368
RNN-1	RNN	2021_ROS_E	3	107	2.3	5.2	PsC2870p236	PsC3195p368
RNN-2	RNN	2021_ROS_E	6b	22	3	15.7	PsC6423p69	PsC27596p426
RNN-2	RNN	2021_ROS_L	7b	117	2.4	7.8	PsC2575p276	PsC3005p202
DTM-1	DTM	2021_SAS_E	3	43	2.8	17.2	V-7	PsC5404p543
DTM-1	DTM	2021_SAS_L	3	77	2.1	22.1	PsC13141p220	PsC5404p543
DTM-2	DTM	2020_LL	2	34	2.4	9.0	PsC2169p461	PsC6145p417
DTM-3	DTM	2020_LL	3	107	2.1	8.5	PsC2870p236	PsC3195p368
DTM-4	DTM	2020_LL	5	74	2.5	8.8	V-11	PsC26393p87
DTM-5	DTM	2021_SAS_L	7b	64	2.4	18.8	PsC4056p224	PsC6058p226
LD-1	LD	2020_LL	7b	62	2.4	22.9	PsC4056p224	PsC6058p226
LD-2	LD	2020_ROS	1	166	2.1	15.2	PsC6221p181	PsC24631p193
LD-3	LD	2020 ROS	5	3	2.1	17.9	PsC20442p333	V-12
LD-4	LD	2021_SAS_L	6a	57	3.3	20.1	PsC26551p132	PsC13805p217
LD-5	LD	2021_ROS_E	5	120	2.2	11.9	PsC26393p87	PsC5316p234
LD-6	LD	2021_ROS_L	1	105	2.4	19.4	PsC2159p286	PsC6329p79
HT-1	HT	2021_SAS_E	3	59	2.1	15.9	PsC4623p176	PsC13141p220
HT-2	HT	2021_SAS_E	5	59	2.6	28.5	V-11	PsC26393p87
yield-1	yield	2021_ROS_L	3	98	3.2	31.7	PsC2870p236	PsC3195p368

Note: For the full trial names refer to Table 5.1; for the full trait names refer to Table 5.2; LG, linkage group; PVE: percentage phenotypic variation explained; CI, confident interval.

Regarding the six pea seed quality traits, various QTLs were associated with seed protein, EE and ash concentration, among which four consistent QTLs were characterized over multiple trials. For protein concentration, one QTL at LG III was identified in two trials, and it explained 27% of the variation of the phenotypic variation among the RILs at 2020 LL and 18% of the variation at 2021_SAS_L (Table 5.7). For EE concentration, one QTL at LG I was characterized in all trials except 2020_LL, and it explained 15%–30% of the phenotypic variation among the five trials. The other consistent QTL for EE concentration was at LG V, and it was identified at 2021_SAS_L and 2021_ROS_L, explaining ~20% of the phenotypic variation at each location. For ash concentration, only one QTL at LG VIa was consistent, and it was detected at 2021_SAS_E, 2021_SAS_L and 2021_ROS_L. No QTL was identified for starch and ADF concentration from any trial data, and three separate QTLs were found associated with NDF at 2021_SAS_E and 2021_SAS_L.

Table 5.7. Summary of quantitative traits loci associated with pea six seed compositions at Saskatoon, Rosthern and Lucky Lake in 2020 and 2021.

QTL symbols	Traits	trials	LG	peak position	LOD	PVE	left marker	right marker
protein-1	protein	2020_LL	3	12	2.3	27.2	V-1	PsC5352p274
protein-1	protein	2021_SAS_L	3	15	2.4	18.1	V-1	PsC5352p274
protein-2	protein	2021_ROS_E	7b	33	3.7	34.4	PsC13180p419	PsC4635p485
protein-3	protein	2021_ROS_L	6a	11	2.5	26.8	PsC14430p66	PsC3267p192
EE-1	EE	2020_ROS	1	70	2.8	30.0	PsC25275p166	PsC2159p286
EE-1	EE	2021_SAS_E	1	79	2.8	28.4	PsC2159p286	PsC6329p79
EE-1	EE	2021_SAS_L	1	81	2.4	17.8	PsC2159p286	PsC6329p79
EE-1	EE	2021_ROS_E	1	72	2.7	16.7	PsC2159p286	PsC6329p79
EE-1	EE	2021_ROS_L	1	71	2	15.3	PsC25275p166	PsC2159p286
EE-2	EE	2021_SAS_L	5	71	3	18.1	V-11	PsC26393p87
EE-2	EE	2021_ROS_L	5	72	2.2	21.5	V-11	PsC26393p87
EE-3	EE	2020_LL	5	106	2.2	2.9	PsC26393p87	PsC5316p234
EE-4	EE	2020_LL	2	35	2.0	2.9	PsC2169p461	PsC6145p417
EE-5	EE	2020_ROS	7b	3	2.1	10.2	PsC27413p94	PsC21060p179
EE-6	EE	2021_ROS_L	3	115	2.3	15	PsC2870p236	PsC3195p368
ash-1	ash	2021_SAS_E	6a	112	4.3	17	PsC28635p290	PsC3875p766
ash-1	ash	2021_SAS_L	6a	112	2.5	25.2	PsC28635p290	PsC3875p766
ash-1	ash	2021_ROS_L	6a	112	2.3	6.4	PsC28635p290	PsC3875p766
ash-2	ash	2021_SAS_E	2	55	2.2	19.1	PsC2169p461	PsC6145p417
ash-3	ash	2021_SAS_E	5	35	3.2	18.6	V-12	V-11
ash-4	ash	2021_ROS_L	1	66	2.5	17	PsC25275p166	PsC2159p286
NDF-1	NDF	2021_SAS_E	5	100	2.1	26.3	PsC26393p87	PsC5316p234
NDF-2	NDF	2021_SAS_E	6a	76	2.9	17.7	PsC26551p132	PsC13805p217
NDF-3	NDF	2021_SAS_L	7a	13	2	10.9	PsC4963p219	PsC4676p597

Note: For the full trial names refer to Table 5.1; for the full trait names refer to Table 5.3; LG, linkage group; PVE: percentage phenotypic variation explained; CI, confident interval.

5.4.6 Selection of stable indices as valid criteria for pea heat tolerance

Ideally, a desirable heat tolerant variety is expected with promising yield potential at both normal and HS environment. In practical studies of crop abiotic stresses, not limited to HS, it was common to see that varieties could have contrasting yield performances between control and stressful environments, and stress tolerant varieties often had relatively high yield at stress environment but relatively lower yield at normal environment. Likewise, the yield data of 2020_ROS (control environment) and 2021_ROS_L (HS environment) among 39 RILs in PR-24

were poorly correlated (R=0.09, Table 5.9). As a result, numerous indices were proposed as other criteria for heat tolerance, instead of yield alone. In this study, five indices were evaluated based on their reliability reported in previous literature. According to HS and precipitation conditions (Table 5.1), yield data at 2020_ROS (Y2020_ROS) were considered as yield potential at control environment, and yield data of 2021_ROS_E (Y2021_ROS_E) and 2021_ROS_L (Y2021_ROS_L) were separately used as yield potential at HS. GMP, SSI, STI, YSI and ATI were calculated for each RIL between 2020_ROS and 2021_ROS_E, and between 2020_ROS and 2021_ROS_L. Among the five indices, only GMP and STI had significantly positive correlations with yield at both control and HS environments (Table 5.8 and 5.9). These two indices could be suitable standards for the selection of pea varieties with consistently high yield potential under both normal and HS environments.

Table 5.8. Correlation summary among yield data of 2020_ROS and 2021_ROS_E and five indices deduced from these two field trials.

	Y2020_ROS	Y2021_ROS_E	GMP	SSI	STI	YSI
Y2021_ROS_E	0.41*					
GMP	0.72***	0.93***				
SSI	0.16ns	-0.83***	-0.56***			
STI	0.71***	0.93***	1.0***	-0.56***		
YSI	-0.16ns	0.83***	0.56***	-1.0***	0.56***	
ATI	0.88***	-0.07ns	0.31ns	0.60***	0.30ns	-0.60***

Note: For the full names of GMP, SSI, STI, YSI and ATI, and their calculation formulas refer to section 5.2.4 ‘Heat tolerant indices calculation’.

Table 5.9. Correlation summary among yield data of 2020_ROS and 2021_ROS_L and five indices deduced from these two field trials.

	Y2020_ROS	Y2021_ROS_L	GMP	SSI	STI	YSI
Y2021_ROS_L	0.09ns					
GMP	0.56***	0.88***				
SSI	0.43***	-0.85***	-0.49***			
STI	0.56***	0.87***	0.99***	-0.48**		
YSI	-0.43**	0.85***	0.49***	-1***	0.48**	
ATI	0.96***	-0.20ns	0.29ns	0.68***	0.29ns	-0.68***

Note: For the full names of GMP, SSI, STI, YSI and ATI, and their calculation formulas refer to section 5.2.4 ‘Heat tolerant indices calculation’.

5.5 Discussion

5.5.1 Trait selection for yield prediction

Field pea is historically known as a cool-season legume crop, but its production has been expanding to drier and hotter prairie regions in North America in the last two decades. Thus, it is important to understand how the pea crop can phenologically adapt to a warming environment. Pea varieties with resistance to HS damage at flowering stage are most desirable, as extremely hot days at anthesis seems to be the major temperature cause for yield reduction (Bueckert et al., 2015; Huang et al., 2017). In our study, 2021 had a much hotter and drier summer than 2020, which caused a sharp yield reduction in 2021 compared with the average yield of RILs in 2020 (Table 5.1).

Selection on agronomic traits that can aid in yield gain is always the core interest in the breeding work. In this study, we examined the relationship between six agronomic traits (i.e., days to flowering, days to maturity, plant height, lodging, reproductive node number on main-stem, and pod number on main-stem) and the plot yield at different HS and soil moisture conditions. Plant height seemed to a useful indictor for yield prediction, as it was positively associated with plot yield in all six trials regardless of temperature and soil moisture conditions (Table 5.4). It is worth noting that all RILs were derived from two semi-leafless pea varieties with good lodging resistance. Semi-leafless leaf type is the commercially desirable leaf type and this trait contributes to reduced lodging and cooler canopy temperature under heat and drought conditions (Tafesse et al., 2019). At controlled heat conditions (2020_LL and 2020_ROS) and heat condition with sufficient soil moisture (2021_SAS_E and 2021_SAS_L), DTF was positively associated with plot yield in PR-24 population, whereas the correlation became negative in the trials where heat and drought were confounded (2021_ROS_E and 2021_ROS_L). A similar trend was previously reported in another RIL population, whose both parents were also semi-leafless pea cultivars (Huang et al., 2017). It seems to be a dilemma for pea breeding; breeders may need to develop relatively late maturing varieties for environments where growing conditions are typically favorable but develop early maturing varieties for growing regions where terminal heat and drought are typically severe.

For the two yield component traits, pod number on the main stem was positively correlated with plot yield in three out of the four HS trials. A similar correlation was also seen in

the other RIL population (Huang et al., 217) and a set of 24 varieties tested under heat stressful environments (Tafesse et al., 2019). Flower abortion is considered as the main reason for yield loss in HS, as a result, peas with better pod set success would potentially yield more under HS. In control environments, the correlation between pod number on the main-stem and plot yield was less significant, presumably because under favorable growth conditions, basal branches are also an important source for seed production (Huang et al., 2017; Singh et al., 2011). Field pea basal branching was complexly affected by genotype, plant density, site-year and their interactions (Spies et al., 2010).

5.5.2 Quantitative trait loci comparison

Linkage analysis, also known as QTL mapping, is a common method for genetic dissection of important agronomic and seed quality traits in field pea (for example, Gali et al., 2018; Huang et al., 2017). In this manner, promising QTLs for DTF, DTM, PN and RNN, were characterized over multiple field trials from this work.

The QTL for DTF was located at LG VII, i.e., chromosome 7, flanking from Chr7_67444481 to Chr7_169190602 (Table 5.6). This QTL explained 20% of the overall DTF variation in the RIL population. One QTL associated with flowering time at LG VII was previously reported by Klein et al. (2014), however, the precise position of these two QTLs was not comparable due to the lack of common markers between the two studies. Consistent QTLs for DTF were also characterized at LG I, III, V and VI in other pea RIL populations from our group, which were derived from various genetic backgrounds (Gali et al., 2018; Huang et al., 2017). What's more, in a recent review paper, Weller and Ortega (2015) summarized 20-plus genome loci that were putatively associated with pea's flowering time and inflorescence. Interestingly, marker 'PsC6058p226' within the QTL 'DTF-1' in this study, was a fraction of gene '7g038920', which was differentially expressed in the anthers of the two parental varieties at HS (Huang et al., 2021). In addition, a cluster of other genes within this QTL were also differentially expressed between the two parental varieties (Appendix F).

In this study, a QTL for PN was identified at LG I, which accounted for 28% of the phenotypic variation at 2021_ROS_L and 15% variation at 2021_SAS_E (Table 5.6). Our finding corroborated previous studies, where stable QTLs for PN were also characterized at this LG (Guindon et al., 2019; Irzykowska et al., 2004; Krajewski et al., 2012). In an association

mapping study relating to 135 diverse pea accessions (Tafesse et al., 2020), six SNP markers on LG I had strong association with PN over multiple site-years, and three of those six markers were within the QTL region described here. Additional QTLs for PN were previously characterized at LG III in the other pea genetic populations (Huang et al., 2017; Klein et al., 2014).

Separate QTLs for DTM and RNN were found at LG III. The QTL for DTM was mapped to a big locus (around 80 cM), partly due to the low marker abundance of the current genetic map. The QTL for RNN was co-localized with the QTL for PN identified at 2020_LL. QTLs for both traits were characterized at LG III in previous linkage mapping studies (Gali et al., 2018; Huang et al., 2017; Jha et al., 2016; Klein et al., 2014; Tar'an et al., 2004). In another association mapping study, which was made up of a set of 92 diverse pea cultivars, several markers at LG III also seemed strongly associated with the number of reproductive nodes (Jiang et al., 2017).

Interestingly, numerous genes within each of the above four stable QTLs were differentially expressed between CDC Amarillo and PR11-2 in chapter 4, where heat responsive genes were characterized for anthers and stipules of the two parental varieties of PR-24. The list of differentially expressed genes at each QTL region are shown in Appendices F–I. Among these, eight genes are highlighted. For example, 5g161560 and 5g171400 within QTL-RNN-1, and 7g093240 within QTL-DTF-1, are involved in the biological process of oxidation-reduction (GO:0055114), which was significantly upregulated in anthers of PR11-2 (heat tolerant), down-regulated in anthers of PR11-90 (heat susceptible), and partially upregulated and partially down-regulated in CDC Amarillo (moderately heat tolerant) in chapter 4 (Fig 4.6). The different response in oxidation-reduction process among the three varieties are proposed to be linked with heat tolerance. Similarly, 7g051680 within QTL-DTF-1 is related to regulation of transcription (GO:0006355), which was only upregulated in stipules of PR11-2. 5g198960 (ATP biosynthetic process, GO:0006754) and 5g165160 (cellulose metabolic process, GO:0030243) within QTL-RNN-1 were respectively downregulated in anthers and stipules of CDC Amarillo. Besides, 7g091560 and 7g091680 within QTL-DTF-1 are related with photosynthesis (GO:0009765), which was downregulated in stipules of CDC Amarillo but not in PR11-2 as reported in chapter 4.0.

5.5.3 Assessment of heat tolerance indices

Tolerance to stress is measured by the performance differential of genotypes between stress and non-stress conditions. Based on yield production, Fernandez (1992) divided genotypes into four groups: a) genotypes with consistently high yield under both stress and control environments; b) genotypes with desirable yield only at control condition; c) genotypes having relatively higher yield at stress condition; d) low yield at both conditions. Several indices for drought stress were suggested by different researchers to distinguish the four groups, and were validated for their effectiveness, for instance, SSI in spring wheat (Fisher and Maurer, 1978) and in pea (Grzesiak et al., 1996), YSI in soybean (Bouslama and Schapaugh, 1984), STI in common bean (Schneider et al., 1997), ATI and SSPI in wheat (Mousavi et al., 2008), and STI and GMP in chickpea (Farshadfar and Geravandi, 2013). Drought often occurs with HS in Canadian pea production regions, as well as in most cool-season legume production areas in other countries. The above indices might be useful for HS in field pea. As a result, they were validated based on two years' yield data at Rosthern in this study, where 2020_ROS was considered as the control environment, 2021_ROS_E and 2021_ROS_L were regarded as two HS environments. Only GMP and STI were positively correlated with yield at control and HS conditions in both sets of data (Table 5.8 and 5.9). GMP and STI were previously reported effective indices to select drought-tolerant chickpea varieties by Farshadfar and Geravandi (2013). Individual top ten RILs with highest GMP and STI were filtered out from 2020_ROS/2021_ROS_E and 2020_ROS/2021_ROS_L, four RILs were common, which were PR-24-03, PR-24-08, PR-24-10 and PR-24-12. These four RILs were considered as best heat tolerant RILs in PR-24 population.

5.6 Conclusion

The thesis general hypothesis III was accepted that flowering and yield related traits were genetically controlled under normal and HS environments. Four QTLs were characterized in PR-24 population over multiple trials, one each for DTF (chromosome 7), DTM (chromosome 5), RNN (chromosome 5), and PN (chromosome 2). These QTLs informed the genetic control of pea reproductive development especially under HS. Plant height and PN could be effective indicators for yield prediction because positive correlations of both traits with plot yield were significant under both control and HS conditions. GMP and STI seem to be suitable criteria for breeding heat tolerant pea varieties with high yield potential under both non-stressful and HS

environments. Based on these two criteria, PR-24-03, PR-24-08, PR-24-10 and PR-24-12 were selected as the most heat tolerant RILs.

Chapter 6.0 GENERAL DISCUSSION AND CONCLUSIONS

Field pea is sensitive to high temperature. The heat susceptibility of pea was worse than other pulse crops including chickpea and lentil, in Australia, where yield loss of pea started at temperatures above 25°C (Sadras et al., 2012). In Saskatchewan, a yield plateau was seen when summer seasonal temperature was 17.5°C and above (Bueckert et al., 2015). Over the last decade, substantial progress has been made in the characterization of heat induced physiological effects on pollen (Jiang et al., 2015; 2019), ovules (Osorio et al., 2022), vegetative traits (Tafesse et al., 2019; 2020; 2021) and pod development (Jiang et al., 2020) in pea. However, the understanding of heat response at the gene level is still scarce, which builds the main focus for this PhD thesis.

6.1 Temperature threshold for pea heat stress

In my PhD thesis, the average yield of PR-24 was significantly reduced in 2021 due to the combined effects of heat and drought, compared with the yield in 2020. Taking the Rosthern data for example, the population yield average of early and late seeding trials at Rosthern was 365 and 233 kg ha^{-1} in 2021, which was only 26% and 16% of the 2020 Rosthern yield average, 1420 kg ha^{-1}. With regards to air temperature thresholds for HS in pea, previous literature indicated a large variation. In controlled environments, Jiang et al. (2015) identified that pollen germination and development was impaired after seven days at 33/18°C, and the damage effect was doubled when the daytime temperature increased to 36°C. The threshold maximum temperature for yield reduction in field trials in the Canadian prairie was closer to 28°C, and the conclusion was drawn based on the negative association between the frequency of hot days with daily maximum temperature ≥28°C in the crop growing season and pea yield over 2000-2009 (Bueckert et al., 2015). Huang et al. (2017) also observed that pea yield was negatively correlated with the mean daily maximum temperature at the flowering stage ($r = –0.79$) ranging from 23°C to 26°C among five field trials in 2013 and 2014, which was in agreement with the finding in Australia that during the critical reproductive phases, grain yield was negatively associated with maximum temperatures over 25°C (Sadras et al., 2012). HS in the field needs a

lower temperature threshold than in controlled environments for the same physiological stage, as other abiotic stresses usually coincide, drought most typically, as well as the much greater light intensity in the field. In this thesis, the average daily maximum temperature from flowering to physiological maturity ranged from 28.9 to 30.0°C among the four trials in 2021, whereas the average daily maximum of the two trials in 2020 was only around 25°C. Therefore, 2021 field trials were typically HS environments, and 2020 trials were normal environments.

6.2 General pea plant heat response at the transcriptional level

In plant cellular defense against HS, the induction of HSP is one of the major responses. HSPs act as molecular chaperones which are proteins that facilitate folding of other functional proteins, especially at the secondary and tertiary structure and prevent them from denaturation and aggregation during exposure to HS. The results of chapter 3.0 and chapter 4.0 suggested that HSP induced heat protection was conserved in both anthers and stipules among heat tolerant and susceptible pea varieties (Fig 3.1, Table 4.4). Heat induced transcription of HSP genes was also generally seen in rice (Chandel et al., 2013) and bread wheat (Mishra et al., 2017), when multiple heat tolerant and susceptible varieties were evaluated. Similar to this study, the differential induction of HSP genes did not depend on the classification of the variety's heat tolerance. Both studies found heat tolerant varieties and susceptible varieties could have strong induction of some HSP genes. One possible explanation for the high HSP induction in a heat susceptible variety is that a rescue mechanism is taking place. Another possible explanation is that response to HS is a quantitative trait affected by many genes and the so-called heat tolerant and heat susceptible varieties do not differ completely in their reaction to HS. As shown in Figure 4.3 from Study II, numerous up/down-regulated genes were common among pea varieties. The transcriptome re-programming and chaperone function of HSPs are considered to contribute to a plant's basal thermal tolerance (Fragkostefanakis et al., 2015).

Transcriptome profiling indicated that genes in biological processes related to cell wall and lipid components were sensitive to heat damage in both anthers and stipules of the three pea varieties (Fig 4.5). Similarly, in heat stressed lentil, a major group of heat responsive genes was involved in plasma membrane and cell wall integrity (Singh et al., 2019). Among the cell wall associated genes, multiple pectin methyl esterase genes were downregulated at HS, and this reduced expression was consistent with the findings in canola (Yu et al., 2014). Pectin, a mixture

of polysaccharides, is a major component in plant cell walls, especially in dicotyledonous plants (Mohnen, 2008). In addition to its adhesive properties, adjustment of pectin content in cell walls is proposed to be associated with various physiological functions during the plant life cycle, as well as contributing to signal transduction to various conditions.

The lipid metabolic process was damaged by HS in both anthers and stipules among all three pea varieties (Figs 4.5 and 4.7), which was also seen in rice heat stressed anthers (Endo et al., 2009). Jiang et al. (2015) proposed that CDC Sage had better pollen germination than CDC Golden at extreme HS (36°C) because lipid composition on the pollen coat and exine of CDC Sage was more stable compared with that of CDC Golden at 36°C. It would be interesting to apply the pollen surface composition profiling assay, that is mid-infrared attenuated total reflectance, into anthers and stipules in this study, to build a further correlation.

6.3 Transcriptomic changes associated with pea heat tolerance variation

Heat induced transcriptional reprogramming was dynamic and varied greatly among pea varieties. PR11-2, the most heat tolerant variety in this research, had the lowest number of heat responsive genes in anthers and stipules among the three varieties, contrastingly, the number of responsive genes in CDC Amarillo, a moderately heat tolerant variety, was the greatest in both plant organs (Fig 4.3). This variation was also seen in maize, where tolerant cultivars S058 and L043 had the most and least abundant heat responsive genes among four tolerant and four susceptible varieties, respectively (Frey et al., 2015). Collectively, it is suggested that plant heat tolerance could be achieved by different mechanisms. PR11-2 had the best ability to maintain transcriptional stability, because it had a much lower number of unique biological processes that were significantly down-regulated, compared with the other varieties (Fig 4.6).

Among a variety's distinct heat responsive biological processes, respiratory electron transport chain (ETC) and cellular response to DNA damage stimulus were commonly upregulated in anthers and stipules of PR11-2 and CDC Amarillo, respectively. To my understanding, upregulation means protection, thus these two biological processes are proposed as common protective mechanisms by which PR11-2 and CDC Amarillo had better heat tolerance compared to PR11-90. In ETC, Psat1g132320 and 6g041400 encoding mitochondrial cytochrome b and Psat1g132440 encoding uncharacterized protein were upregulated. Cytochrome b-c1 complex is an essential component of the mitochondrial ETC. Chilling induced

accumulation of reactive oxygen species resulting from the downregulation of ETC led to oxidative stress (Hu et al., 2008). For cellular response to DNA damage stimulus in stipules, gene accession 2g148040 (DNA mismatch repair protein MLH3), 5g135640 (DNA excision repair protein), 6g105320 (cryptochrome 2b), and 6g199840 (DNA mismatch repair protein MSH3), were commonly up-regulated in PR11-2 and CDC Amarillo. The putative functions of the four genes were involved with three DNA repair pathways, but these pathways were well studied in UV light induced stress (Manova and Gruszka, 2015). This result in HS adds one more piece of evidence that a plant's ability to maintain its genome integrity is likely to play a role in its abiotic stress tolerance.

6.4 Endogenous hormone response at heat stress

HS altered normal hormonal regulation in the reproductive development of food legumes, and the reduced concentration and/or signalling of auxin, gibberellin and ethylene at HS led to male sterility, and subsequently impaired the fruit and seed set (Ozga et al., 2017). The information on the involvement of ABA in HS is lacking. Study I, for the first time, characterized ABA homeostasis of pea stipules among different hours of HS, and the results revealed that ABA homeostasis could respond quickly at 3 h HS (Fig 3.2). At 3 h HS, heat tolerant varieties, CDC Meadow and PR11-2 had greater ABA responses than heat susceptible varieties, Nitouche and PR11-90 in terms of 25% faster ABA turnover rate. This response differential between tolerant and susceptible varieties linked well with different heat tolerance of the four varieties at the field level (Table 3.1). Similarly, a faster ABA turnover was reported in a heat tolerant rice variety (Tang et al., 2008), a cold-tolerant rice variety (Oliver et al., 2007), and two drought tolerant wheat varieties (Ji et al., 2011) compared to their susceptible checks. What's more, results of Study II demonstrated that the transcription of multiple genes associated with auxin, ethylene, and ABA was altered at HS. In PR11-2 stipule, response to hormone (GO:0009725) was significantly upregulated (Fig 4.6). Two gene accessions, 7g124320 and 7g124440, were particularly interesting. Both genes putatively encode ABA 8'-hydroxylase and were significantly up-regulated at HS. The transcriptional upregulation of these two genes linked well with the observed ABA turnover via C-8' hydroxylation in study I, and these two genes are thus proposed to be the candidate genes.

6.5 Traits associated with pea heat tolerance

The transcriptional response was complex, and the responsive genes were characterized to be a few thousands (Fig 4.3). The dynamic transcription response further complicated the genetic control of field traits, e.g., days to flowering (DTF), which are quantitative traits affected by multiple genes under average field conditions. Especially for yield, no stable QTL was detected in PR-24 population in the HS trials. Similarly, in another pea RIL population, namely PR11, no stable QTL associated with plot yield was found either (Huang et al., 2017). Three stable QTLs were still successfully identified, i.e., QTL-DTF1 on chromosome 7, QTL-PN1 at chromosome 2 and QTL-RNN1 at chromosome 5 (Table 5.6). Klein et al. (2014) also reported one QTL associated with flowering duration and termination on chromosome 7, however, the precise position of these two QTLs was not comparable due to the lack of common markers between the two studies. The identified QTL-PN1 corroborated previous studies, where stable QTLs for pod number were also characterized on chromosome 2 (Guindon et al., 2019; Irzykowska et al., 2004; Krajewski et al., 2012). In an association mapping study relating to 135 diverse pea accessions (Tafesse et al., 2020), six SNP markers had strong associations with pod number over multiple site-years, and three of those six markers were within QTL-PN1 described here. For reproductive node number, one QTL on chromosome 5 was previously reported by Huang et al. (2017) in the other pea population. Notedly, eight genes (5g161560, 5g165160, 5g171400, 5g198960, 7g051680, 7g091560, 7g091680 and 7g093240) within the aforementioned QTLs were differentially expressed between two parental varieties of PR-24 in the transcriptome study chapter of the thesis; as a result, these eight genes were proposed to contribute to the superior heat tolerance of PR11-2 over CDC Amarillo. Genetic markers for these genes could be developed for further validation on a broader range of pea genotypes.

Traits such as leaf surface wax, chlorophyll concentration, and reproductive stem length were found responsive to differing extents of heat and drought stresses in a genetically diverse pea population (Tafesse et al., 2020; 2021), however, the researchers did not further elucidate the likely relationship of these heat responsive traits with plot yield, which is generally the core interest in practical pea breeding. The correlations among other agronomic traits with yield can be contrasting between normal and heat stressful environments as seen in this thesis. Only plant height was positively associated with plot yield in all six trials; thus, it seems to be a useful indicator for yield prediction regardless of temperature and soil moisture conditions (Table 5.4).

Under average western Canadian summer weather conditions, pea varieties with relatively late maturity, taller height and less lodging tend to have higher yields. Under heat, but less droughty conditions, a longer time to flowering period is still desirable for yield gain. When both heat and drought are severe, the correlation between pod number and plot yield is the most positively significant, and similar correlation was also seen in the other population (Huang et al., 2017). Tafesse et al. (2019) concluded that semi-leafless pea varieties were more heat tolerant than normal leaf-type pea varieties due to better lodging resistance. This positive correlation between lodging resistance and yield was statistically significant among 39 RILs of PR-24 in all three trials at Rosthern over 2020-2021.

Yield performance of crop varieties can vary greatly among different stress environments, and correlation results in Table 5.9 showed that plot yield among RILs of PR-24 did not correlate between 2020 Rosthern, control trial, and 2021 Rosthern late seeding trial, that is a HS trial ($r = 0.09$). Based on yield production, Fernandez (1992) divided genotypes into four groups: a) genotypes with consistently high yield under both stress and control environments; b) genotypes with desirable yield only at control condition; c) genotypes having relatively higher yield at stress condition; d) low yield at both conditions. Several indices for drought stress were suggested by different researchers to distinguish the four groups, and were validated for their effectiveness, for instance, stress susceptibility index in pea (Grzesiak et al., 1996), yield stability index in soybean (Bouslama and Schapaugh, 1984), stress tolerance index (STI) in common bean (Schneider et al., 1997), and STI and geometric mean productivity (GMP) in chickpea (Farshadfar and Geravandi, 2013). Drought often occurs with HS in Canadian pea production regions, as well as in most cool-season legume production areas in other countries. The aforementioned indices were thus validated based on yield data of Rosthern in 2020 and 2021. Only GMP and STI were positively correlated with yield under control and HS conditions in both sets of data (Table 5.8 and 5.9). GMP and STI were previously reported effective indices to select drought-tolerant chickpea varieties by Farshadfar and Geravandi (2013). The ten RILs with highest GMP and STI were filtered out from 2020_ROS/2021_ROS_E and 2020_ROS/2021_ROS_L, and four RILs were common, which were PR-24-03, PR-24-08, PR-24-10 and PR-24-12. These four RILs were considered as the most heat tolerant RILs in PR-24 population. This study showed that both GMP and STI could be effective criteria for pea heat tolerance assessment.

6.6 Conclusions

HSP mediated heat protection was conserved among two pairs of pea varieties in Study I and was further validated in Study II. ABA homeostasis was firstly characterized in pea stipules among two pairs of pea varieties, and at 3 h HS, heat tolerant varieties had a higher ABA turnover rate via C'8 hydroxylation than the heat susceptible counterpart. The heat induced transcription of two putative ABA 8'-hydroxylase genes, 7g124320 and 7g124440, was further identified in Study II, thus these two genes are proposed to be candidate genes for ABA mediated heat response. Besides HSP response, a number of additional heat responses were profiled, which greatly expands the previous understanding in pea heat response. My thesis highlighted that the biological processes relating to cell wall and lipid integrity were generally sensitive to heat damage. Furthermore, the transcriptomic responsive variation was characterized among PR11-2 (best heat tolerant), CDC Amarillo (intermediate heat tolerant), and PR11-90 (worst heat tolerant). Biological processes i.e., cellular response to DNA damage stimulus in stipules, and electron transport chain in anthers, were only observed in heat induced PR11-2 and CDC Amarillo, and their relevance to field pea heat tolerance is worth further validation. A genetic population, PR-24, derived from the cross of PR11-2 and CDC Amarillo was tested between normal and heat stress trials in 2020 and 2021. Only plant height was positively correlated with plot yield in all six trials of Study III. Under normal field conditions, DTF and DTM were positively correlated with plot yield. Under HS conditions, positive correlation between pod number and plot yield was the most significant association. Four stable QTLs were identified, which were QTL-DTF at chromosome 7, QTL-PN at chromosome 2 and both QTL-DTM and QTL-RNN at chromosome 5. Numerous genes within these QTLs overlapped with heat responsive genes in Study II, among which eight genes were further proposed to contribute to the superior heat tolerance of PR11-2 over CDC Amarillo.

6.7 Future prospects

The thesis provides substantial information related to the understanding of transcriptional heat response in pea, complex interactions between genotypes and environments, and underlying plant genetic architecture. The following further research is recommended.

1. This thesis research proves that HSP induced heat protection is conserved at the transcriptional level. Expression response at the protein level still needs to be characterized. A classical assay is

Western blotting. One alternative method is a higher throughput proteome profiling via liquid chromatography-mass spectrometry.

2. ABA homeostasis of pea stipules was found to be rapidly responsive to heat stress. It is worth discovering ABA mediated heat response of anthers or other reproductive organs because ABA and other plant hormones are important in the regulation of reproductive development. The requirement to grow a substantial number of plants for sufficient sample collection should be noted.

3. Genes relating to lipid metabolism, transport and localization, are generally downregulated in stipules and anthers of three pea varieties in this study. It would be interesting to conduct high throughput lipid-omics profiling.

4. The genetic map of PR-24 in this thesis could be improved for mapping resolution by including additional genetic markers by conducting genotyping-by-sequencing. The improved genetic mapping would increase the accuracy of QTL detection.

5. Results of correlation analysis and identified QTLs from PR-24 could be validated in other populations with different genetic background.

References

Abass, M., & Rajashekar, C. (1993). Abscisic acid accumulation in leaves and cultured cells during heat acclimation in grapes. Horticulture science, 28(1), 50-52.

Abdelrahman, M., El-Sayed, M., Jogaiah, S., Burritt, D. J., & Tran, L.-S. P. (2017). The "STAY-GREEN" trait and phytohormone signaling networks in plants under heat stress. Plant cell reports, 36(7), 1009-1025.

Abeysingha GLDN. 2015. The effects of auxins on seed yield parameters in wheat, pea and canola grown under controlled environmental and western Canadian field conditions. MSc thesis, University of Alberta, Edmonton, AB.

Agarwal, M., Katiyar-Agarwal, S., Sahi, C., Gallie, D. R., & Grover, A. (2001). *Arabidopsis thaliana* Hsp100 proteins: kith and kin. Cell stress & chaperones, 6(3), 219.

Ahmed, F. E., Hall, A. E., & DeMason, D. A. (1992). Heat injury during floral development in cowpea (*Vigna unguiculata*, Fabaceae). American journal of botany, 79(7), 784-791.

Alves-Carvalho, S., Aubert, G., Carrère, S., Cruaud, C., Brochot, A. L., Jacquin, F., . . . Kreplak, J. (2015). Full-length de novo assembly of RNA-seq data in pea (P isum sativum L.) provides a gene expression atlas and gives insights into root nodulation in this species. The plant journal, 84(1), 1-19.

Arganosa, G., Warkentin, T., Racz, V., Blade, S., Phillips, C., & Hsu, H. (2006). Prediction of crude protein content in field peas using near infrared reflectance spectroscopy. Canadian journal of plant science, 86(1), 157-159.

Baldwin, L., Domon, J.-M., Klimek, J. F., Fournet, F., Sellier, H., Gillet, F., . . . Rayon, C. (2014). Structural alteration of cell wall pectins accompanies pea development in response to cold. Phytochemistry, 104, 37-47.

Baron, K. N., Schroeder, D. F., & Stasolla, C. (2012). Transcriptional response of abscisic acid (ABA) metabolism and transport to cold and heat stress applied at the reproductive stage of development in Arabidopsis thaliana. Plant science, 188, 48-59.

Bharti, K., von Koskull-Döring, P., Bharti, S., Kumar, P., Tintschl-Körbitzer, A., Treuter, E., & Nover, L. (2004). Tomato heat stress transcription factor HsfB1 represents a novel type of general transcription coactivator with a histone-like motif interacting with the plant CREB binding protein ortholog HAC1. The plant cell, 16(6), 1521-1535.

Bogdanov, I. V., Shenkarev, Z. O., Finkina, E. I., Melnikova, D. N., Rumynskiy, E. I., Arseniev, A. S., & Ovchinnikova, T. V. (2016). A novel lipid transfer protein from the pea *Pisum sativum*: isolation, recombinant expression, solution structure, antifungal activity, lipid binding, and allergenic properties. BMC plant biology, 16(1), 107.

Bouslama, M., & Schapaugh, W. (1984). Stress tolerance in soybean. Part 1: evaluation of three screening techniques for heat and drought tolerance. Crop science. 24: 933-937.

Buchfink, B., Xie, C., & Huson, D. H. (2015). Fast and sensitive protein alignment using DIAMOND. Nature methods, 12(1), 59.

Bueckert, R. A., & Clarke, J. M. (2013). Annual crop adaptation to abiotic stress on the Canadian prairies: Six case studies. Canadian journal of plant science, 93(3), 375-385.

Bueckert, R. A., Wagenhoffer, S., Hnatowich, G., & Warkentin, T. D. (2015). Effect of heat and precipitation on pea yield and reproductive performance in the field. Canadian journal of plant science, 95(4), 629-639.

Bush, E. and Lemmen, D.S., editors (2019): Canada's Changing Climate Report; Government of Canada, Ottawa, ON. 444 p.

Chandel, G., Dubey, M., & Meena, R. (2013). Differential expression of heat shock proteins and heat stress transcription factor genes in rice exposed to different levels of heat stress. Journal of plant biochemistry and biotechnology, 22(3), 277-285.

Chen, H., Osuna, D., Colville, L., Lorenzo, O., Graeber, K., Kuester, H., . . . Kranner, I. (2013). Transcriptome-wide mapping of pea seed ageing reveals a pivotal role for genes related to oxidative stress and programmed cell death. PLoS One, 8(10).

Chidambaranathan, P., Jagannadham, P. T. K., Satheesh, V., Kohli, D., Basavarajappa, S. H., Chellapilla, B., . . . Srinivasan, R. (2018). Genome-wide analysis identifies chickpea (Cicer arietinum) heat stress transcription factors (Hsfs) responsive to heat stress at the pod development stage. Journal of plant research, 131(3), 525-542.

Clark, T. A., Sugnet, C. W., & Ares, M. (2002). Genomewide analysis of mRNA processing in yeast using splicing-specific microarrays. Science, 296(5569), 907-910.

Cutler, A. J., & Krochko, J. E. (1999). Formation and breakdown of ABA. Trends in plant science, 4(12), 472-478.

DeMason, D. A., & Polowick, P. L. (2009). Patterns of DR5: GUS expression in organs of pea (*Pisum sativum*). International journal of plant sciences, 170(1), 1-11.

DeRocher, A., & Vierling, E. (1995). Cytoplasmic HSP70 homologues of pea: differential expression in vegetative and embryonic organs. Plant molecular biology, 27(3), 441-456.

DeRocher, A. E., Helm, K. W., Lauzon, L. M., & Vierling, E. (1991). Expression of a conserved family of cytoplasmic low molecular weight heat shock proteins during heat stress and recovery. Plant physiology, 96(4), 1038-1047.

Die, J. V., Román, B., Nadal, S., & González-Verdejo, C. I. (2010). Evaluation of candidate reference genes for expression studies in *Pisum sativum* under different experimental conditions. Planta, 232(1), 145-153.

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., . . . Gingeras, T. R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics, 29(1), 15-21.

Duarte, J., Rivière, N., Baranger, A., Aubert, G., Burstin, J., Cornet, L., . . . Pichon, J.-P. (2014). Transcriptome sequencing for high throughput SNP development and genetic mapping in pea. BMC genomics, 15(1), 126.

Endo, M., Tsuchiya, T., Hamada, K., Kawamura, S., Yano, K., Ohshima, M., . . . Kawagishi-Kobayashi, M. (2009). High temperatures cause male sterility in rice plants with transcriptional alterations during pollen development. Plant and cell physiology, 50(11), 1911-1922.

Environment Canada. (2013). Climate trends and variations bulletin-2013 annual. Environment Canada Catalogue no: En81-23E-PDF.

Environment Canada. (2014). Climate trends and variations bulletin-summer 2014. Environment Canada Catalogue no: En81-23E-PDF.

FAOSTAT. (2021). Food and Agriculture Organization of the United Nations Statistics Division.

Farshadfar, E., & Geravandi, M. (2013). Repeatability of drought tolerance indices in chickpea genotypes over stress and non-stress environments. Acta agronomica hungarica, 61(2), 123-137.

Fernandez, G. C. (1992). Effective selection criteria for assessing plant stress tolerance. Paper presented at the Proceeding of the International Symposium on Adaptation of Vegetables and other Food Crops in Temperature and Water Stress, Aug. 13-16, Shanhua, Taiwan, 1992.

Fischer, R., & Maurer, R. (1978). Drought resistance in spring wheat cultivars. I. Grain yield responses. Australian journal of agricultural research, 29(5), 897-912.

Fondevilla, S., Küster, H., Krajinski, F., Cubero, J. I., & Rubiales, D. (2011). Identification of genes differentially expressed in a resistant reaction to Mycosphaerella pinodes in pea using microarray technology. BMC genomics, 12(1), 1-15.

Fragkostefanakis, S., Röth, S., Schleiff, E., & Scharf, K. D. (2015). Prospects of engineering thermotolerance in crops through modulation of heat stress transcription factor and heat shock protein networks. Plant, cell & environment, 38(9), 1881-1895.

Fragkostefanakis, S., Simm, S., El-Shershaby, A., Hu, Y., Bublak, D., Mesihovic, A., . . . Theres, K. (2019). The repressor and co-activator HsfB1 regulates the major heat stress transcription factors in tomato. Plant, cell & environment, 42(3), 874-890.

Franssen, S. U., Shrestha, R. P., Bräutigam, A., Bornberg-Bauer, E., & Weber, A. P. (2011). Comprehensive transcriptome analysis of the highly complex *Pisum sativum* genome using next generation sequencing. BMC genomics, 12(1), 227.

Frey, F. P., Urbany, C., Hüttel, B., Reinhardt, R., & Stich, B. (2015). Genome-wide expression profiling and phenotypic evaluation of European maize inbreds at seedling stage in response to heat stress. BMC genomics, 16(1), 123.

Gali, K. K., Liu, Y., Sindhu, A., Diapari, M., Shunmugam, A. S., Arganosa, G., . . . Tar'an, B. (2018). Construction of high-density linkage maps for mapping quantitative trait loci for multiple traits in field pea (Pisum sativum L.). BMC plant biology, 18(1), 1-25.

Giorno, F., Wolters-Arts, M., Mariani, C., & Rieu, I. (2013). Ensuring reproduction at high temperatures: the heat stress response during anther and pollen development. Plants, 2(3), 489-506.

González-Schain, N., Dreni, L., Lawas, L. M., Galbiati, M., Colombo, L., Heuer, S., . . . Kater, M. M. (2016). Genome-wide transcriptome analysis during anthesis reveals new insights into the molecular basis of heat stress responses in tolerant and sensitive rice varieties. Plant and cell physiology, 57(1), 57-68.

Grzesiak, S., Filek, W., Pienkowski, S., & Nizioł, B. (1996). Screening for drought resistance: Evaluation of drought susceptibility index of legume plants under natural growth conditions. Journal of agronomy and crop science, 177(4), 237-244.

Guilioni, L., Wéry, J., & Lecoeur, J. (2003). High temperature and water deficit may reduce seed number in field pea purely by decreasing plant growth rate. Functional plant biology, 30(11), 1151-1164.

Guilioni, L., Wery, J., & Tardieu, F. (1997). Heat stress-induced abortion of buds and flowers in pea: is sensitivity linked to organ age or to relations between reproductive organs? Annals of botany, 80(2), 159-168.

Guindon, M. F., Martin, E., Cravero, V., Gali, K. K., Warkentin, T. D., & Cointry, E. (2019). Linkage map development by GBS, SSR, and SRAP techniques and yield-related QTLs in pea. Molecular breeding, 39(4), 1-16.

Hahn, A., Bublak, D., Schleiff, E., & Scharf, K.-D. (2011). Crosstalk between Hsp90 and Hsp70 chaperones and heat stress transcription factors in tomato. The plant cell, 23(2), 741-755.

Hall, A. E. (1992). Breeding for heat tolerance. Plant breeding reviews, 10(2), 129-168.

Hasegawa, K., Kamada, S., Takehara, S., Takeuchi, H., Nakamura, A., Satoh, S., & Iwai, H. (2020). Rice Putative Methyltransferase Gene OsPMT16 Is Required for Pistil Development Involving Pectin Modification. Frontiers in plant science, 11, 475.

Hays, D. B., Do, J. H., Mason, R. E., Morgan, G., & Finlayson, S. A. (2007). Heat stress induced ethylene production in developing wheat grains induces kernel abortion and increased maturation in a susceptible cultivar. Plant science, 172(6), 1113-1123.

Hedhly, A. (2011). Sensitivity of flowering plant gametophytes to temperature fluctuations. Environmental and experimental botany, 74, 9-16.

Henriet, C., Aimé, D., Térézol, M., Kilandamoko, A., Rossin, N., Combes-Soia, L., . . . Kreplak, J. (2019). Water stress combined with sulfur deficiency in pea affects yield components but mitigates the effect of deficiency on seed globulin composition. Journal of experimental botany, 70(16), 4287-4304.

Hernandez, L. D., & Vierling, E. (1993). Expression of low molecular weight heat-shock proteins under field conditions. Plant physiology, 101(4), 1209-1216.

Higashi, Y., Okazaki, Y., Myouga, F., Shinozaki, K., & Saito, K. (2015). Landscape of the lipidome and transcriptome under heat stress in *Arabidopsis thaliana*. Scientific reports, 5(1), 1-11.

Higashitani, A. (2013). High temperature injury and auxin biosynthesis in microsporogenesis. Frontiers in plant science, 4, 47.

Hoegh-Guldberg, O., Jacob, D., Bindi, M., Brown, S., Camilloni, I., Diedhiou, A., . . . Guiot, J. (2018). Impacts of 1.5°C global warming on natural and human systems. Global warming of 1.5 C. An IPCC Special Report.

Hu, W., Song, X., Shi, K., Xia, X., Zhou, Y., & Yu, J. (2008). Changes in electron transport, superoxide dismutase and ascorbate peroxidase isoenzymes in chloroplasts and mitochondria of cucumber leaves as influenced by chilling. Photosynthetica, 46(4), 581.

Huang, S., Gali, K. K., Lachagari, R. V., Chakravartty, N., Bueckert, R. A., Tar'an, B., & Warkentin, T. D. (2021). Identification of heat responsive genes in pea stipules and anthers through transcriptional profiling. PLoS One, 16(11), e0251167.

Huang, S., Gali, K. K., Tar'an, B., Warkentin, T. D., & Bueckert, R. A. (2017). Pea phenology: crop potential in a warming environment. Crop Science, 57(3), 1540-1551.

Ikeda, M., Mitsuda, N., & Ohme-Takagi, M. (2011). Arabidopsis HsfB1 and HsfB2b act as repressors of the expression of heat-inducible Hsfs but positively regulate the acquired thermotolerance. Plant physiology, 157(3), 1243-1254.

Irzykowska, L., & Wolko, B. (2004). Interval mapping of QTLs controlling yield-related traits and seed protein content in *Pisum sativum*. Journal of applied genetics, 45(3), 297-306.

Iturriaga, E. A., Leech, M. J., Barratt, D. P., & Wang, T. L. (1994). Two ABA-responsive proteins from pea (Pisum sativum L.) are closely related to intracellular pathogenesis-related proteins. Plant molecular biology, 24(1), 235-240.

Jeuffroy, M., Duthion, C., Meynard, J., & Pigeaire, A. (1990). Effect of a short period of high day temperatures during flowering on the seed number per pod of pea (*Pisum sativum* L). Agronomie, 10(2), 139-145.

Ji, X., Dong, B., Shiran, B., Talbot, M. J., Edlington, J. E., Hughes, T., . . . Dolferus, R. (2011). Control of abscisic acid catabolism and abscisic acid homeostasis is important for reproductive stage stress tolerance in cereals. Plant physiology, 156(2), 647-662.

Jiang, Y., Diapari, M., Bueckert, R. A., Tar'an, B., & Warkentin, T. D. (2017). Population structure and association mapping of traits related to reproductive development in field pea. Euphytica, 213(9), 1-20.

Jiang, Y., Lahlali, R., Karunakaran, C., Kumar, S., Davis, A. R., & Bueckert, R. A. (2015). Seed set, pollen morphology and pollen surface composition response to heat stress in field pea. Plant, cell & environment, 38(11), 2387-2397.

Jiang, Y., Lahlali, R., Karunakaran, C., Warkentin, T. D., Davis, A. R., & Bueckert, R. A. (2019). Pollen, ovules, and pollination in pea: Success, failure, and resilience in heat. Plant, cell & environment, 42(1), 354-372.

Jiang, Y., Lindsay, D. L., Davis, A. R., Wang, Z., MacLean, D. E., Warkentin, T. D., & Bueckert, R. A. (2020). Impact of heat stress on pod-based yield components in field pea (*Pisum sativum* L.). Journal of agronomy and crop science, 206(1), 76-89.

Jha, A. B., Tar'an, B., Stonehouse, R., & Warkentin, T. D. (2016). Identification of QTLs associated with improved resistance to ascochyta blight in an interspecific pea recombinant inbred line population. Crop science, 56(6), 2926-2939.

Kalra, N., Chakraborty, D., Sharma, A., Rai, H., Jolly, M., Chander, S., . . . Mittal, R. (2008). Effect of increasing temperature on yield of some winter crops in northwest India. Current science, 82-88.

Klein, A., Houtin, H., Rond, C., Marget, P., Jacquin, F., Boucherot, K., . . . Lejeune-Hénaut, I. (2014). QTL analysis of frost damage in pea suggests different mechanisms involved in frost tolerance. Theoretical and applied genetics, 127(6), 1319-1330.

Krajewski, P., Bocianowski, J., Gawłowska, M., Kaczmarek, Z., Pniewski, T., Święcicki, W., & Wolko, B. (2012). QTL for yield components and protein content: a multienvironment study of two pea (*Pisum sativum* L.) populations. Euphytica, 183(3), 323-336.

Kreplak, J., Madoui, M.-A., Cápal, P., Novak, P., Labadie, K., Aubert, G., . . . Main, D. (2019). A reference genome for pea provides insight into legume genome evolution. Nature genetics, 51(9), 1411-1422.

Krishna, P., & Gloor, G. (2001). The Hsp90 family of proteins in Arabidopsis thaliana. Cell stress & chaperones, 6(3), 238.

Kurepin, L. V., Qaderi, M. M., Back, T. G., Reid, D. M., & Pharis, R. P. (2008). A rapid effect of applied brassinolide on abscisic acid concentrations in Brassica napus leaf tissue subjected to short-term heat stress. Plant growth regulation, 55(3), 165-167.

Kumar, S., Kaushal, N., Nayyar, H., & Gaur, P. (2012). Abscisic acid induces heat tolerance in chickpea (*Cicer arietinum* L.) seedlings by facilitated accumulation of osmoprotectants. Acta physiologiae plantarum, 34(5), 1651-1658.

Lahlali, R., Jiang, Y., Kumar, S., Karunakaran, C., Liu, X., Borondics, F., . . . Bueckert, R. (2014). ATR–FTIR spectroscopy reveals involvement of lipids and proteins of intact pea pollen grains to heat stress tolerance. Frontiers in plant science, 5, 747.

Lai, C.-P., Huang, L.-M., Chen, L.-F. O., Chan, M.-T., & Shaw, J.-F. (2017). Genome-wide analysis of GDSL-type esterases/lipases in Arabidopsis. Plant molecular biology, 95(1), 181-197.

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. Nature methods, 9(4), 357.

Larkindale, J., Hall, J. D., Knight, M. R., & Vierling, E. (2005). Heat stress phenotypes of Arabidopsis mutants implicate multiple signaling pathways in the acquisition of thermotolerance. Plant physiology, 138(2), 882-897.

Lenne, C., Block, M., Garin, J., & Douce, R. (1995). Sequence and expression of the mRNA encoding HSP22, the mitochondrial small heat-shock protein in pea leaves. Biochemical journal, 311(3), 805-813.

Li, X., Li, Y., Qu, M., Xiao, H., Feng, Y., Liu, J., . . . Yu, M. (2016). Cell wall pectin and its methyl-esterification in transition zone determine Al resistance in cultivars of pea (*Pisum sativum*). Frontiers in plant science, 7, 39.

Lin, Y., Cheng, Y., Jin, J., Jin, X., Jiang, H., Yan, H., & Cheng, B. (2014). Genome duplication and gene loss affect the evolution of heat shock transcription factor genes in legumes. PLoS One, 9(7), e102825.

Lin, B., Wang, J., Liu, H.-C., Chen, R.-W., Meyer, Y., Barakat, A., & Delseny, M. (2001). Genomic analysis of the Hsp70 superfamily in *Arabidopsis thaliana*. Cell stress & chaperones, 6(3), 201.

Ling, H. (2008). Sequence analysis of GDSL lipase gene family in *Arabidopsis thaliana*. Pakistan journal of biological sciences: PJBS, 11(5), 763-767.

Liu, H. C., Liao, H. T., & Charng, Y. Y. (2011). The role of class A1 heat shock factors (HSFA1s) in response to heat and other stresses in Arabidopsis. Plant, cell & environment, 34(5), 738-751.

Liu, Z., Xin, M., Qin, J., Peng, H., Ni, Z., Yao, Y., & Sun, Q. (2015). Temporal transcriptome profiling reveals expression partitioning of homeologous genes contributing to heat and drought acclimation in wheat (*Triticum aestivum* L.). BMC plant biology, 15(1), 152.

Lobell, D. B., & Field, C. B. (2007). Global scale climate–crop yield relationships and the impacts of recent warming. Environmental research letters, 2(1), 014002.

Lucau-Danila, A., Toitot, C., Goulas, E., Blervacq, A.-S., Hot, D., Bahrman, N., . . . Delbreil, B. (2012). Transcriptome analysis in pea allows to distinguish chilling and acclimation mechanisms. Plant physiology and biochemistry, 58, 236-244.

Luo, Q. (2011). Temperature thresholds and crop production: a review. Climatic change, 109(3), 583-598.

Mahoney, J. (1991). Field pea. New crops: Agronomy and potential of alternative crop species, 53-62.

Mangelsen, E., Kilian, J., Harter, K., Jansson, C., Wanke, D., & Sundberg, E. (2011). Transcriptome analysis of high-temperature stress in developing barley caryopses: early stress responses and effects on storage compound biosynthesis. Molecular plant, 4(1), 97-115.

Manova, V., & Gruszka, D. (2015). DNA damage and repair in plants–from models to crops. Frontiers in plant science, 6, 885.

Mayfield, J. A., Fiebig, A., Johnstone, S. E., & Preuss, D. (2001). Gene families from the Arabidopsis thaliana pollen coat proteome. Science, 292(5526), 2482-2485.

McDonald, G., & Paulsen, G. (1997). High temperature effects on photosynthesis and water relations of grain legumes. Plant and soil, 196(1), 47-58.

Meng, L., Li, H., Zhang, L., & Wang, J. (2015). QTL IciMapping: integrated software for genetic linkage map construction and quantitative trait locus mapping in biparental populations. The crop journal, 3(3), 269-283.

Metzker, M. L. (2010). Sequencing technologies—the next generation. Nature reviews genetics, 11(1), 31-46.

Mishra, D., Shekhar, S., Agrawal, L., Chakraborty, S., & Chakraborty, N. (2017). Cultivar-specific high temperature stress responses in bread wheat (Triticum aestivum L.) associated with physicochemical traits and defense pathways. Food chemistry, 221, 1077-1087.

Mishra, D., Shekhar, S., Singh, D., Chakraborty, S., & Chakraborty, N. (2018). Heat shock proteins and abiotic stress tolerance in plants Regulation of heat shock protein responses (pp. 41-69): Springer.

Mohnen, D. (2008). Pectin structure and biosynthesis. Current opinion in plant biology, 11(3), 266-277.

Mousavi, S., YAZDI, S. B., Naghavi, M., Zali, A., Dashti, H., & Pourshahbazi, A. (2008). Introduction of new indices to identify relative drought tolerance and resistance in wheat genotypes. Desert, 12(2): 165-178.

Munier-Jolain, N., Biarnes, V., & Chaillet, I. (2010). Physiology of the pea crop: CRC Press.

Nisa, M.-U., Huang, Y., Benhamed, M., & Raynaud, C. (2019). The plant DNA damage response: Signaling pathways leading to growth inhibition and putative role in response to stress conditions. Frontiers in plant science, 10, 653.

Nover, L., Bharti, K., Döring, P., Mishra, S. K., Ganguli, A., & Scharf, K.D. (2001). Arabidopsis and the heat stress transcription factor world: how many heat stress transcription factors do we need? Cell stress & chaperones, 6(3), 177.

Ofir, M., Gross, Y., Bangerth, F., & Kigel, J. (1993). High temperature effects on pod and seed production as related to hormone levels and abscission of reproductive structures in common bean (*Phaseolus vulgaris* L.). Scientia horti culturae, 55(3-4), 201-211.

Ohama, N., Sato, H., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2017). Transcriptional regulatory network of plant heat stress response. Trends in plant science, 22(1), 53-65.

Oliver, S. N., Dennis, E. S., & Dolferus, R. (2007). ABA regulates apoplastic sugar transport and is a potential signal for cold-induced pollen sterility in rice. Plant and cell physiology, 48(9), 1319-1330.

Osorio, E. E., Davis, A. R., & Bueckert, R. A. (2022). High temperatures disturb ovule development in field pea (*Pisum sativum*). Botany, 100(1), 47-61.

Ozga, J. A., Kaur, H., Savada, R. P., & Reinecke, D. M. (2017). Hormonal regulation of reproductive growth under normal and heat-stress conditions in legume and other model crop species. Journal of Experimental Botany, 68(8), 1885-1894.

Paul, P. J., Samineni, S., Thudi, M., Sajja, S. B., Rathore, A., Das, R. R., . . . Varshney, R. (2018). Molecular mapping of QTLs for heat tolerance in chickpea. International journal of molecular sciences, 19(8), 2166.

Petkova, V., Nikolova, V., Kalapchieva, S., Stoeva, V., Topalova, E., & Angelova, S. (2008). Physiological response and pollen viability of Pisum sativum genotypes under high temperature influence. Paper presented at the IV Balkan Symposium on Vegetables and Potatoes 830.

Porch, T., & Jahn, M. (2001). Effects of high-temperature stress on microsporogenesis in heat-sensitive and heat-tolerant genotypes of *Phaseolus vulgaris*. Plant, cell & environment, 24(7), 723-731.

Qin, D., Wu, H., Peng, H., Yao, Y., Ni, Z., Li, Z., . . . Sun, Q. (2008). Heat stress-responsive transcriptome analysis in heat susceptible and tolerant wheat (Triticum aestivum L.) by using Wheat Genome Array. BMC genomics, 9(1), 432.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., . . . Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids research, 41(D1), D590-D596.

Ridge, P., & Pye, D. (1985). The effects of temperature and frost at flowering on the yield of peas grown in a Mediterranean environment. Field crops research, 12, 339-346.

Sadras, V., Lake, L., Chenu, K., McMurray, L., & Leonforte, A. (2012). Water and thermal regimes for field pea in Australia and their implications for breeding. Crop and pasture science, 63(1), 33-44.

Sah, S. K., Reddy, K. R., & Li, J. (2016). Abscisic acid and abiotic stress tolerance in crop plants. Frontiers in plant science, 7, 571.

Salminen, T. A., Blomqvist, K., & Edqvist, J. (2016). Lipid transfer proteins: classification, nomenclature, structure, and function. Planta, 244(5), 971-997.

Sarkar, N. K., Kundnani, P., & Grover, A. (2013). Functional analysis of Hsp70 superfamily proteins of rice (*Oryza sativa*). Cell stress and chaperones, 18(4), 427-437.

Saskatchewan specialty crop report. (2020). https://publications.saskatchewan.ca/#/products/111437.

Savada, R. P., Ozga, J. A., Jayasinghege, C. P., Waduthanthri, K. D., & Reinecke, D. M. (2017). Heat stress differentially modifies ethylene biosynthesis and signaling in pea floral and fruit tissues. Plant molecular biology, 95(3), 313-331.

Scharf, K.-D., Berberich, T., Ebersberger, I., & Nover, L. (2012). The plant heat stress transcription factor (Hsf) family: structure, function and evolution. Biochimica et Biophysica Acta (BBA)-gene regulatory mechanisms, 1819(2), 104-119.

Scharf, K.D., Siddique, M., & Vierling, E. (2001). The expanding family of Arabidopsis thaliana small heat stress proteins and a new family of proteins containing α-crystallin domains (Acd proteins). Cell stress & chaperones, 6(3), 225.

Schena, M., Shalon, D., Davis, R. W., & Brown, P. O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science, 270(5235), 467-470.

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C T method. Nature protocols, 3(6), 1101.

Schneider, K. A., Rosales-Serna, R., Ibarra-Perez, F., Cazares-Enriquez, B., Acosta-Gallegos, J. A., Ramirez-Vallejo, P., . . . Kelly, J. D. (1997). Improving common bean performance under drought stress. Crop science, 37(1), 43-50.

Schubert, M., Lindgreen, S., & Orlando, L. (2016). AdapterRemoval v2: rapid adapter trimming, identification, and read merging. BMC research notes, 9(1), 88.

Shah, Z., Iqbal, A., Khan, F. U., Khan, H. U., Durrani, F., & Ahmad, M. Z. (2020). Genetic manipulation of pea (*Pisum sativum* L.) with Arabidopsis's heat shock factor HsfA1d improves ROS scavenging system to confront thermal stress. Genetic resources and crop evolution, 1-9.

Shanmugavadivel, P., Sv, A. M., Prakash, C., Ramkumar, M., Tiwari, R., Mohapatra, T., & Singh, N. K. (2017). High resolution mapping of QTLs for heat tolerance in rice using a 5K SNP array. Rice, 10(1), 28.

Siddique, K. (1999). Abiotic stresses of cool season pulses in Australia. Agriculture Western Australia, and Centre for Legumes in Mediterranean Agriculture, University of Western Australia: Nedlands, WA.

Sindhu, A., Ramsay, L., Sanderson, L.-A., Stonehouse, R., Li, R., Condie, J., . . . Diapari, M. (2014). Gene-based SNP discovery and genetic mapping in pea. Theoretical and applied genetics, 127(10), 2225-2241.

Singh, A., Singh, S., & Babu, J. D. P. (2011). Heritability, character association and path analysis studies in early segregating population of field pea (*Pisum sativum* L. var. arvense). International journal of plant breeding and genetics, 5(1), 86-92.

Singh, D., Singh, C. K., Singh Tomar, R. S., & Pal, M. (2017). Genetics and molecular mapping of heat tolerance for seedling survival and pod set in lentil. Crop science, 57(6), 3059-3067.

Singh, D., Singh, C. K., Taunk, J., Jadon, V., Pal, M., & Gaikwad, K. (2019). Genome wide transcriptome analysis reveals vital role of heat responsive genes in regulatory mechanisms of lentil (*Lens culinaris* Medikus). Scientific reports, 9(1), 1-19.

Soares-Cavalcanti, N. M., Belarmino, L. C., Kido, E. A., Pandolfi, V., Marcelino-Guimarães, F. C., Rodrigues, F. A., . . . Benko-Iseppon, A. M. (2012). Overall picture of expressed Heat Shock Factors in *Glycine max*, *Lotus japonicus* and *Medicago truncatula*. Genetics and molecular biology, 35(1), 247-259.

Spies, J. M., Warkentin, T., & Shirtliffe, S. (2010). Basal branching in field pea cultivars and yield-density relationships. Canadian journal of plant science, 90(5), 679-690.

Srikanthbabu, V., Krishnaprasad, B. T., Gopalakrishna, R., Savitha, M., & Udayakumar, M. (2002). Identification of pea genotypes with enhanced thermotolerance using temperature induction response technique (TIR). Journal of plant physiology, 159(5), 535-545.

Srivastava, S., Rahman, M. H., Shah, S., & Kav, N. N. (2006). Constitutive expression of the pea ABA-responsive 17 (ABR17) cDNA confers multiple stress tolerance in Arabidopsis thaliana. Plant biotechnology journal, 4(5), 529-549.

Sudheesh, S., Sawbridge, T. I., Cogan, N. O., Kennedy, P., Forster, J. W., & Kaur, S. (2015). De novo assembly and characterisation of the field pea transcriptome using RNA-Seq. BMC genomics, 16(1), 611.

Swindell, W. R., Huebner, M., & Weber, A. P. (2007). Transcriptional profiling of Arabidopsis heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways. BMC genomics, 8(1), 125.

Tafesse, E. G., Gali, K. K., Lachagari, V., Bueckert, R., & Warkentin, T. D. (2020). Genome-wide association mapping for heat stress responsive traits in field pea. International journal of molecular sciences, 21(6), 2043.

Tafesse, E. G., Gali, K. K., Lachagari, V. B. R., Bueckert, R., & Warkentin, T. D. (2021). Genome-Wide Association Mapping for Heat and Drought Adaptive Traits in Pea. Genes, 12(12), 1897.

Tafesse, E. G., Warkentin, T. D., & Bueckert, R. A. (2019). Canopy architecture and leaf type as traits of heat resistance in pea. Field crops research, 241, 107561.

Tang, R.S., Zheng, J.C., Jin, ZQ., Zhang, D.D., Huang, Y.H., & Chen, L.G. (2008). Possible correlation between high temperature-induced floret sterility and endogenous levels of IAA, GAs and ABA in rice (*Oryza sativa* L.). Plant growth regulation, 54(1), 37-43.

Tar'an, B., Warkentin, T., Somers, D., Miranda, D., Vandenberg, A., Blade, S., & Bing, D. (2004). Identification of quantitative trait loci for grain yield, seed protein concentration and maturity in field pea (*Pisum sativum* L.). Euphytica, 136(3), 297-306.

Thévenin, J., Pollet, B., Letarnec, B., Saulnier, L., Gissot, L., Maia-Grondard, A., . . . Jouanin, L. (2011). The simultaneous repression of CCR and CAD, two enzymes of the lignin biosynthetic pathway, results in sterility and dwarfism in *Arabidopsis thaliana*. Molecular plant, 4(1), 70-82.

Tian, T., Liu, Y., Yan, H., You, Q., Yi, X., Du, Z., . . . Su, Z. (2017). agriGO v2. 0: a GO analysis toolkit for the agricultural community, 2017 update. Nucleic acids research, 45(W1), W122-W129.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., . . . Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature protocols, 7(3), 562-578.

Usadel, B., Poree, F., Nagel, A., Lohse, M., Czedik-Eysenberg, A., & Stitt, M. (2009). A guide to using MapMan to visualize and compare Omics data in plants: a case study in the crop species, Maize. Plant, cell & environment, 32(9), 1211-1229.

Wahid, A., Gelani, S., Ashraf, M., & Foolad, M. R. (2007). Heat tolerance in plants: an overview. Environmental and experimental botany, 61(3), 199-223.

Wang, F., Zang, X., Kabir, M. R., Liu, K., Liu, Z., Ni, Z., . . . Peng, H. (2014). A wheat lipid transfer protein 3 could enhance the basal thermotolerance and oxidative stress resistance of Arabidopsis. Gene, 550(1), 18-26.

Wang, T. L., Donkin, M. E., & Martin, E. S. (1984). The physiology of a wilty pea: abscisic acid production under water stress. Journal of experimental botany, 35(8), 1222-1232.

Wang, W., Vinocur, B., Shoseyov, O., & Altman, A. (2004). Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends in plant science, 9(5), 244-252.

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. Nature reviews genetics, 10(1), 57-63.

Warkentin, T., Vandenberg, A., Banniza, S., Barlow, B., & Ife, S. (2006). CDC Sage field pea. Canadian journal of plant science, 86(1), 161-162.

Warkentin, T., Vandenberg, A., Tar'an, B., Barlow, S., & Ife, S. (2007). CDC Centennial field pea. Canadian journal of plant science, 87(4), 907-908.

Warkentin, T., Vandenberg, A., Tar'an, B., Barlow, S., & Ife, S. (2007). CDC Meadow field pea. Canadian journal of plant science, 87(4), 909-910.

Warkentin, T., Vandenberg, A., Tar'an, B., Banniza, S., Arganosa, G., Barlow, B., . . . Thompson, M. (2014). CDC Amarillo yellow field pea. Canadian journal of plant science, 94(8), 1539-1541.

Weller, J. L., & Ortega, R. (2015). Genetic control of flowering time in legumes. Frontiers in plant science, 6, 207.

Wu, H.-C., Bulgakov, V. P., & Jinn, T.-L. (2018). Pectin methylesterases: Cell wall remodeling proteins are required for plant response to heat stress. Frontiers in plant science, 9, 1612.

Xue, G.-P., Sadat, S., Drenth, J., & McIntyre, C. L. (2014). The heat shock factor family from *Triticum aestivum* in response to heat and other major abiotic stresses and their role in regulation of heat shock protein genes. Journal of experimental botany, 65(2), 539-557.

Ye, S., Yu, S., Shu, L., Wu, J., Wu, A., & Luo, L. (2012). Expression profile analysis of 9 heat shock protein genes throughout the life cycle and under abiotic stress in rice. Chinese science bulletin, 57(4), 336-343.

Yoshida, T., Ohama, N., Nakajima, J., Kidokoro, S., Mizoi, J., Nakashima, K., . . . Todaka, D. (2011). Arabidopsis HsfA1 transcription factors function as the main positive regulators in heat shock-responsive gene expression. Molecular genetics and genomics, 286(5), 321-332.

Yoshida, T., Sakuma, Y., Todaka, D., Maruyama, K., Qin, F., Mizoi, J., . . . Yamaguchi-Shinozaki, K. (2008). Functional analysis of an Arabidopsis heat-shock transcription factor HsfA3 in the transcriptional cascade downstream of the DREB2A stress-regulatory system. Biochemical and biophysical research communications, 368(3), 515-521.

Yu, E., Fan, C., Yang, Q., Li, X., Wan, B., Dong, Y., . . . Zhou, Y. (2014). Identification of heat responsive genes in Brassica napus siliques at the seed-filling stage through transcriptional profiling. PLoS One, 9(7), e101914.

Zaman, M. S. U., Malik, A. I., Erskine, W., & Kaur, P. (2019). Changes in gene expression during germination reveal pea genotypes with either “quiescence” or “escape” mechanisms of waterlogging tolerance. Plant, cell & environment, 42(1), 245-258.

Zeevaart, J. A., & Creelman, R. A. (1988). Metabolism and physiology of abscisic acid. Annual review of plant physiology and plant molecular biology, 39(1), 439-473.

Zhang, J., & Davies, W. (1987). ABA in roots and leaves of flooded pea plants. Journal of Experimental botany, 38(4), 649-659.

Zhang, J., & Zhang, X. (1994). Can early wilting of old leaves account for much of the ABA accumulation in flooded pea plants? Journal of experimental botany, 45(9), 1335-1342.

Zhang, X., Li, J., Liu, A., Zou, J., Zhou, X., Xiang, J., . . . Chen, X. (2012). Expression profile in rice panicle: insights into heat response mechanism at reproductive stage. PLoS One, 7(11), e49652.

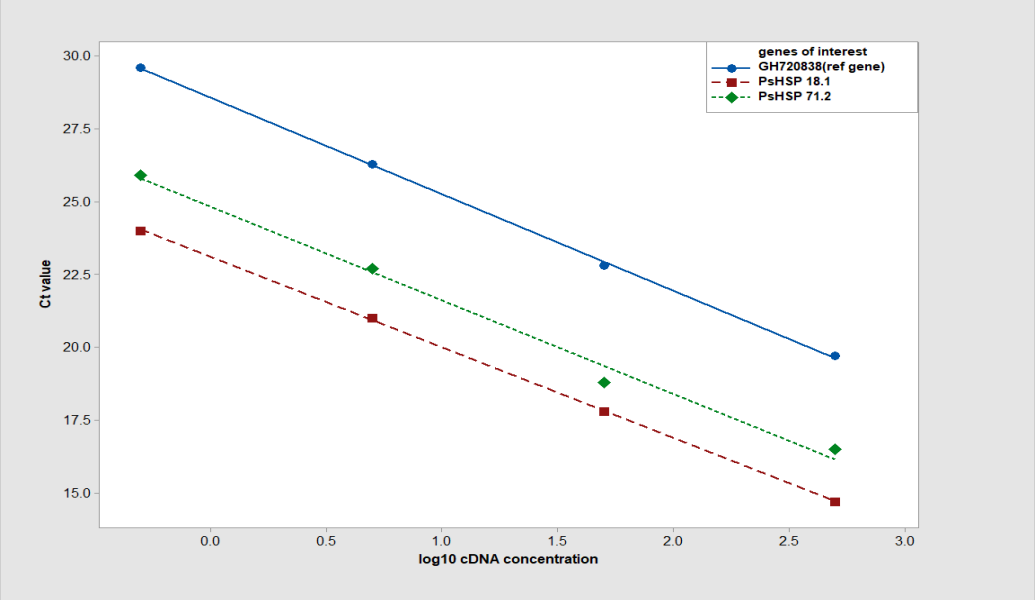
Zhao, Q., Nakashima, J., Chen, F., Yin, Y., Fu, C., Yun, J., . . . Dixon, R. A. (2013). Laccase is necessary and nonredundant with peroxidase for lignin polymerization during vascular development in Arabidopsis. The plant cell, 25(10), 3976-3987.

Zhao, Q., Tobimatsu, Y., Zhou, R., Pattathil, S., Gallego-Giraldo, L., Fu, C., . . . Chen, F. (2013). Loss of function of cinnamyl alcohol dehydrogenase 1 leads to unconventional lignin and a temperature-sensitive growth defect in *Medicago truncatula*. Proceedings of the National Academy of Sciences, 110(33), 13660-13665.

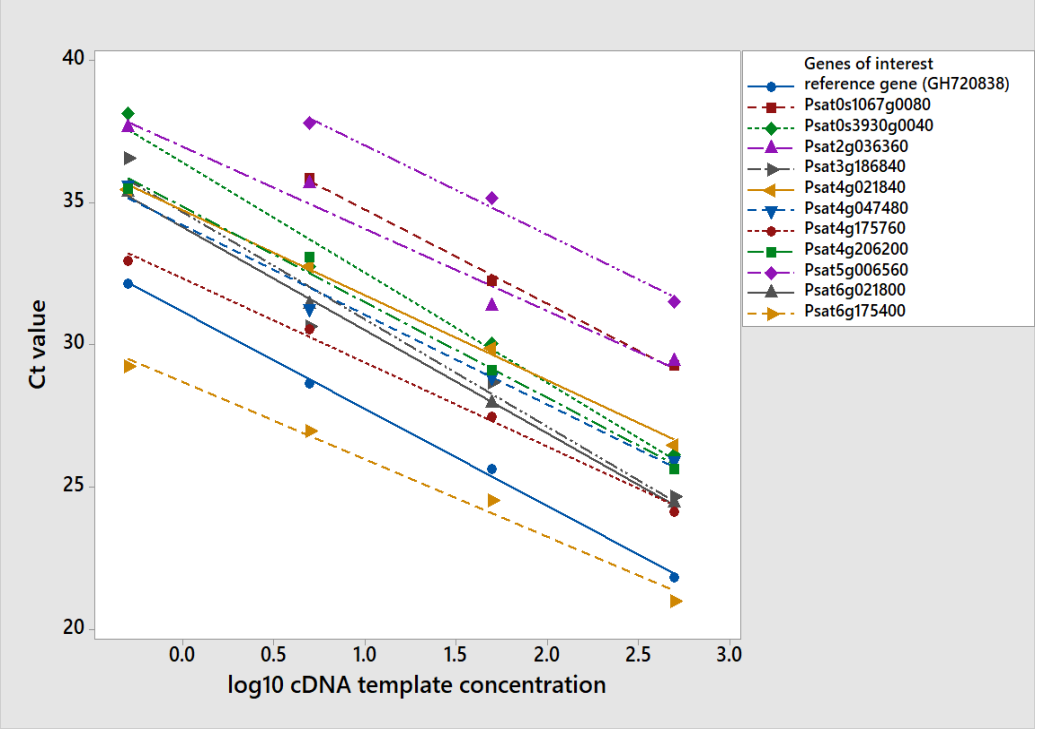
Zhu, B., Ye, C., Lü, H., Chen, X., Chai, G., Chen, J., & Wang, C. (2006). Identification and characterization of a novel heat shock transcription factor gene, GmHsfA1, in soybeans (*Glycine max*). Journal of plant research, 119(3), 247-256.

APPENDICES

Appendix A. Standard curves of primer efficiencies of *PsHSP18.1*, *PsHSP71.2* and reference gene.



Appendix B. qPCR primer efficiency standard curves.



1983 Appendix C. Over-representative GO terms with significance p value in the anther's consistently up-regulated genes among PR11-2,
1984 CDC Amarillo and PR11-90.

Plant organ	biological process	GO term	number	p value
anther	protein folding	GO:0006457	21	2.5E-09
	embryo development	GO:0009790	9	4.3E-06
	multicellular organismal process	GO:0032501	17	6.2E-06
	response to heat	GO:0009408	5	1.6E-04
	multicellular organism development	GO:0007275	15	2.4E-04
	galactose metabolic process	GO:0006012	4	5.3E-04
	regulation of transcription, DNA-templated	GO:0006355	43	7.9E-04
	regulation of cellular metabolic process	GO:0031323	44	8.8E-04
	regulation of RNA metabolic process	GO:0051252	26	9.4E-04
	regulation of gene expression	GO:0010468	44	9.4E-04
	regulation of macromolecule biosynthetic process	GO:0010556	43	9.8E-04
	regulation of nitrogen compound metabolic process	GO:0051171	43	9.8E-04
	regulation of cellular biosynthetic process	GO:0031326	43	1.0E-03
	regulation of biosynthetic process	GO:0009889	43	1.0E-03
	fruit development	GO:0010154	5	1.1E-03
	cellular respiration	GO:0045333	6	1.2E-03
	ATP synthesis coupled electron transport	GO:0042773	4	1.5E-03
	regulation of metabolic process	GO:0019222	45	1.6E-03
	RNA metabolic process	GO:0016070	32	1.7E-03
	regulation of macromolecule metabolic process	GO:0060255	44	1.8E-03
	regulation of primary metabolic process	GO:0080090	43	2.1E-03
	developmental process involved in reproduction	GO:0003006	6	2.1E-03
	energy derivation by oxidation of organic compounds	GO:0015980	6	2.5E-03

	transcription, DNA-templated	GO:0006351	28	2.7E-03
	reproductive process	GO:0022414	6	2.7E-03
	reproduction	GO:0000003	6	2.7E-03
	macromolecule modification	GO:0043412	22	4.6E-03
	respiratory electron transport chain	GO:0022904	4	4.9E-03
	reproductive structure development	GO:0048608	5	7.0E-03
	response to temperature stimulus	GO:0009266	4	7.9E-03
	protein folding	GO:0006457	13	3.2E-05
	response to heat	GO:0009408	4	5.2E-04
	cellular protein modification process	GO:0006464	12	1.7E-03
	carbohydrate metabolic process	GO:0005975	8	2.9E-03
	post-translational protein modification	GO:0043687	12	4.9E-03
	transcription, DNA-templated	GO:0006351	21	5.2E-03
stipule	RNA biosynthetic process	GO:0032774	19	5.3E-03
	phosphate-containing compound metabolic process	GO:0006796	11	5.7E-03
	phosphorus metabolic process	GO:0006793	11	5.8E-03
	regulation of RNA metabolic process	GO:0051252	18	6.6E-03
	phosphorylation	GO:0016310	10	6.6E-03
	metabolic process	GO:0008152	117	8.2E-03
	ATP synthesis coupled proton transport	GO:0015986	4	9.0E-03

1989 Appendix D. Genes in GO:0055114, oxidation-reduction process was upregulated in PR11-2 and CDC Amarillo or down-regulated in
1990 PR11-90 and CDC Amarillo.

Genome locus	Putative function
	Up-regulation in PR11-2 and CDC Amarillo
Psat0s3560g0080	NADH-ubiquinone oxidoreductase chain 1 (EC 7.1.1.2)
Psat0s3689g0040	Cinnamyl alcohol dehydrogenase
Psat1g132320	Ubiquinol-cytochrome-c reductase complex cytochrome b subunit
Psat1g132440	uncharacterized protein
Psat2g113840	Pyruvate dehydrogenase E1 component subunit alpha (EC 1.2.4.1)
Psat4g196000	Myo-inositol oxygenase (Putative inositol oxygenase) (EC 1.13.99.1)
Psat6g041400	Ubiquinol-cytochrome-c reductase complex cytochrome b subunit
Psat7g109520	Putative tetrahydroberberine oxidase (EC 1.3.3.8)
	Down-regulation in PR11-90 and CDC Amarillo
Psat0s2493g0120	Oxidoreductase/ferric-chelate reductase
Psat0s2919g0040	Cytochrome P450 family protein (Putative cytochrome P450)
Psat0s2922g0120	cytochrome P450 CYP736A12-like
Psat0s337g0200	Ty3/gypsy retrotransposon protein
Psat0s3850g0080	probable L-gulonolactone oxidase 6
Psat1g140600	Cytokinin oxidase/dehydrogenase 3-like protein
Psat1g140640	Cytokinin oxidase/dehydrogenase 3-like protein
Psat1g165920	Putative tetrahydroberberine oxidase (EC 1.3.3.8)
Psat1g165960	Putative tetrahydroberberine oxidase (EC 1.3.3.8)
Psat2g180800	Leucoanthocyanidin dioxygenase
Psat3g115040	Cytochrome P450 family protein (Putative geraniol 8-hydroxylase) (EC 1.14.14.83)
Psat3g150200	cytochrome P450 714C2-like isoform X1
Psat4g100080	Naringenin 3-dioxygenase (Putative flavanone 3-dioxygenase) (EC 1.14.11.9)
Psat4g222800	Cytochrome P450 family protein
Psat5g145840	Isoleucine N-monooxygenase 2-like protein
Psat5g148560	Isoleucine N-monooxygenase 2-like protein
Psat5g308280	Ent-kaurene oxidase

	Psat6g135960	Cytochrome P450 family 71 protein (Putative premnaspirodiene oxygenase) (EC 1.14.14.151)

Appendix E. Overlapping of heat responsive genes between our study and Tafesse et al. (2020).

Trait	Locus ID	Gene annotation	PR11-2	PR11-90	CDC Amarillo	PR11-2	PR11-90	CDC Amarillo
			anther			stipule		
SPAD	Psat5g221440	Amidohydrolase ytcj-like protein						
	Psat5g224400	Cysteine-rich receptor-like protein kinase 25						
	Psat5g303840	Putative gamma-glutamylcyclotransferase						
	Psat5g303760	Uncharacterized protein						
CT	Psat5g169800	ABC transporter C family member 3-like						
RSL	Psat5g303680	Putative sterile alpha motif/pointed domain-c						
	Psat7g013080	Aldehyde dehydrogenase family 2 member C						
	Psat7g057040	tRNA (Cytosine(34)-C(5))-methyltransferase						
PN	Psat2g144160	Pectin acetylesterase (EC 3.1.1.-)						
	Psat2g166560	PI-PLC X domain-containing protein						
	Psat2g166520	Putative rapid ALkalinization Factor						
	Psat2g004960	Putative calcium-transporting ATPase						

Note: Trait names, SPAD: Soil plant analysis development meter for the estimation of leaf chlorophyll concentration, CT: canopy temperature, RSL: reproductive stem length, PN: pod number. Red cell represents up-regulated gene expression at HS in this study, whereas blue cell represents a down-regulation.

Appendix F. Accession names of differentially expressed genes between CDC Amarillo and PR11-2 in Chapter 4.0, that are within the consistent QTL for DTF, namely 'DTF-1', in PR-24.

CDC Amarillo				PR11-2			
stipule		anther		stipule		anther	
up-regulation	down-regulation	up-regulation	down-regulation	up-regulation	down-regulation	up-regulation	down-regulation
Psat7g040400	Psat7g041480	Psat7g038920	Psat7g041480	Psat7g040240	Psat7g042160	Psat7g039960	Psat7g040200
Psat7g047880	Psat7g045480	Psat7g039320	Psat7g052800	Psat7g045640	Psat7g045600	Psat7g051680	Psat7g045600
Psat7g049280	Psat7g045840	Psat7g045960	Psat7g065480	Psat7g056640	Psat7g046880	Psat7g058200	Psat7g052000
Psat7g054080	Psat7g047440	Psat7g049360	Psat7g067400	Psat7g080360	Psat7g053720	Psat7g058640	Psat7g058680
Psat7g055440	Psat7g048520	Psat7g051760	Psat7g068160		Psat7g054720	Psat7g063920	Psat7g068240
Psat7g057040	Psat7g052320	Psat7g053920	Psat7g093560		Psat7g063080	Psat7g073560	Psat7g076280
Psat7g057760	Psat7g053160	Psat7g058760	Psat7g094680		Psat7g072720	Psat7g076480	Psat7g081360
Psat7g058760	Psat7g053840	Psat7g061840	Psat7g096560		Psat7g072920	Psat7g093240	Psat7g088600
Psat7g062080	Psat7g054800	Psat7g063000	Psat7g100440		Psat7g081360	Psat7g102800	Psat7g090280
Psat7g067320	Psat7g057960	Psat7g065920	Psat7g100720		Psat7g091320	Psat7g102920	Psat7g090560
Psat7g068200	Psat7g059720	Psat7g071560			Psat7g091520		Psat7g091720
Psat7g068400	Psat7g060640	Psat7g071600			Psat7g102120		Psat7g102520
Psat7g073920	Psat7g064920	Psat7g073960			Psat7g102400		
Psat7g076000	Psat7g065160	Psat7g076600					
Psat7g079560	Psat7g066320	Psat7g079560					
Psat7g079800	Psat7g067280	Psat7g079760					
Psat7g080040	Psat7g070400	Psat7g082920					
Psat7g080400	Psat7g073400	Psat7g087880					
Psat7g080520	Psat7g073960	Psat7g090640					
Psat7g087400	Psat7g075120	Psat7g091680					
Psat7g088200	Psat7g075560	Psat7g091760					
Psat7g090320	Psat7g075640	Psat7g091880					
Psat7g090720	Psat7g075920	Psat7g092000					
Psat7g092280	Psat7g077080	Psat7g093760					
Psat7g092800	Psat7g081600	Psat7g094840					
Psat7g096720	Psat7g082200	Psat7g097600					

Psat7g098200	Psat7g083040	Psat7g100320
Psat7g098440	Psat7g085560	
Psat7g099080	Psat7g085960	
Psat7g100800	Psat7g091560	
Psat7g101640	Psat7g091680	
Psat7g102560	Psat7g097400	
	Psat7g099720	
	Psat7g099800	
	Psat7g100520	
	Psat7g100720	

Appendix G. Accession names of differentially expressed genes between CDC Amarillo and PR11-2 in Chapter 4.0, that are within the consistent QTL for PN, namely 'PN-1', in PR-24.

CDC Amarillo				PR11-2			
stipule		anther		stipule		anther	
up-regulation	down-regulation	up-regulation	down-regulation	up-regulation	down-regulation	up-regulation	down-regulation
Psat2g121760	Psat2g123120	Psat2g122360	Psat2g130280	Psat2g136800	Psat2g124800	Psat2g128240	Psat2g128960
Psat2g125600	Psat2g130280	Psat2g127000	Psat2g150360	Psat2g149240	Psat2g124840	Psat2g141560	Psat2g132560
Psat2g127840	Psat2g130840	Psat2g127040			Psat2g130640	Psat2g144080	Psat2g151400
Psat2g130360	Psat2g131920	Psat2g131200			Psat2g145560	Psat2g146680	Psat2g153120
Psat2g131040	Psat2g134000	Psat2g131480			Psat2g147240	Psat2g149600	Psat2g153680
Psat2g131480	Psat2g137120	Psat2g131520			Psat2g150040	Psat2g155640	Psat2g154560
Psat2g131520	Psat2g138960	Psat2g131680					
Psat2g133320	Psat2g139600	Psat2g140040					
Psat2g137720	Psat2g139720	Psat2g140680					
Psat2g140200	Psat2g142000	Psat2g141680					
Psat2g140960	Psat2g143880	Psat2g142080					
Psat2g141040	Psat2g152360	Psat2g142680					
Psat2g141480	Psat2g155640	Psat2g150600					

Psat2g142040	Psat2g155960	Psat2g154080	
Psat2g142080		Psat2g154160	
Psat2g142680			
Psat2g143320			
Psat2g146720			
Psat2g152320			
Psat2g154000			
Psat2g154160			

Appendix H. Accession names of differentially expressed genes between CDC Amarillo and PR11-2 in Chapter 4.0, that are within the consistent QTL for RNN, namely 'RNN-1', in PR-24.

CDC Amarillo				PR11-2			
stipule		anther		stipule		anther	
up-regulation	down-regulation	up-regulation	down-regulation	up-regulation	down-regulation	up-regulation	down-regulation
Psat5g111040	Psat5g114560	Psat5g110680	Psat5g111400	Psat5g111880	Psat5g111360	Psat5g111880	Psat5g111360
Psat5g113360	Psat5g115160	Psat5g111040	Psat5g115160	Psat5g112280	Psat5g136800	Psat5g113720	Psat5g145640
Psat5g114720	Psat5g119480	Psat5g112360	Psat5g117160	Psat5g119080	Psat5g139000	Psat5g120640	Psat5g151640
Psat5g115640	Psat5g121960	Psat5g112840	Psat5g122240	Psat5g130760	Psat5g140880	Psat5g127080	Psat5g162760
Psat5g116200	Psat5g125800	Psat5g120080	Psat5g125240	Psat5g134560	Psat5g143080	Psat5g130760	
Psat5g118360	Psat5g127360	Psat5g120800	Psat5g133000	Psat5g155760	Psat5g150720	Psat5g141360	
Psat5g119520	Psat5g129480	Psat5g121280	Psat5g133200	Psat5g192680	Psat5g177400	Psat5g151840	
Psat5g128080	Psat5g133800	Psat5g123320	Psat5g136920		Psat5g186800	Psat5g151880	
Psat5g128120	Psat5g139760	Psat5g127280	Psat5g145840			Psat5g155760	
Psat5g128240	Psat5g142440	Psat5g128320	Psat5g148560			Psat5g161200	
Psat5g128480	Psat5g143400	Psat5g130840	Psat5g151600			Psat5g161560	
Psat5g129000	Psat5g143480	Psat5g133680	Psat5g161560			Psat5g170760	
Psat5g130840	Psat5g144840	Psat5g145240	Psat5g164720			Psat5g171400	
Psat5g131960	Psat5g145720	Psat5g149200	Psat5g169200			Psat5g174880	
Psat5g133360	Psat5g147600	Psat5g150600	Psat5g187880			Psat5g179280	

Psat5g133680	Psat5g149520	Psat5g153480	Psat5g193360
Psat5g134320	Psat5g149840	Psat5g163160	Psat5g194880
Psat5g137120	Psat5g150040	Psat5g163320	
Psat5g139680	Psat5g150080	Psat5g163960	
Psat5g144920	Psat5g152320	Psat5g165320	
Psat5g145760	Psat5g153560	Psat5g170120	
Psat5g148160	Psat5g154040	Psat5g170400	
Psat5g150840	Psat5g155840	Psat5g174760	
Psat5g152600	Psat5g156240	Psat5g177360	
Psat5g153880	Psat5g157240	Psat5g177840	
Psat5g154600	Psat5g157800	Psat5g182840	
Psat5g154920	Psat5g158080	Psat5g189160	
Psat5g157440	Psat5g158720	Psat5g189240	
Psat5g162040	Psat5g160600	Psat5g195320	
Psat5g162600	Psat5g161560	Psat5g200040	
Psat5g163320	Psat5g163160		
Psat5g164000	Psat5g164040		
Psat5g165320	Psat5g165160		
Psat5g165440	Psat5g167040		
Psat5g168480	Psat5g168640		
Psat5g169920	Psat5g170400		
Psat5g174440	Psat5g171040		
Psat5g174480	Psat5g172400		
Psat5g175680	Psat5g172440		
Psat5g176320	Psat5g176360		
Psat5g176720	Psat5g177200		
Psat5g178520	Psat5g177560		
Psat5g179560	Psat5g177960		
Psat5g179600	Psat5g181600		
Psat5g182360	Psat5g183640		
Psat5g185760	Psat5g183760		
Psat5g190720	Psat5g187880		
Psat5g198600	Psat5g189200		

Psat5g180400
Psat5g199600

Psat5g190640 Psat5g190680 Psat5g198960	

Appendix I. Accession names of differentially expressed genes between CDC Amarillo and PR11-2 in Chapter 4.0, that are within the consistent QTL for DTM, namely 'DTM-1', in PR-24.

CDC Amarillo				PR11-2			
stipule		anther		stipule		anther	
up-regulation	down-regulation	up-regulation	down-regulation	up-regulation	down-regulation	up-regulation	down-regulation
Psat5g128080	Psat5g125800	Psat5g123320	Psat5g125240	Psat5g130760	Psat5g136800	Psat5g127080	Psat5g145640
Psat5g128120	Psat5g127360	Psat5g127280	Psat5g133000	Psat5g134560	Psat5g139000	Psat5g130760	Psat5g151640
Psat5g128240	Psat5g129480	Psat5g128320	Psat5g133200	Psat5g155760	Psat5g140880	Psat5g141360	Psat5g162760
Psat5g128480	Psat5g133800	Psat5g130840	Psat5g136920	Psat5g192680	Psat5g143080	Psat5g151840	Psat5g206680
Psat5g129000	Psat5g139760	Psat5g133680	Psat5g145840	Psat5g225480	Psat5g150720	Psat5g151880	Psat5g210800
Psat5g130840	Psat5g142440	Psat5g145240	Psat5g148560		Psat5g177400	Psat5g155760	Psat5g210840
Psat5g131960	Psat5g143400	Psat5g149200	Psat5g151600		Psat5g186800	Psat5g161200	Psat5g214400
Psat5g133360	Psat5g143480	Psat5g150600	Psat5g161560		Psat5g210760	Psat5g161560	Psat5g227320
Psat5g133680	Psat5g144840	Psat5g153480	Psat5g164720		Psat5g210800	Psat5g170760	Psat5g235600
Psat5g134320	Psat5g145720	Psat5g163160	Psat5g169200		Psat5g210840	Psat5g171400	Psat5g244000
Psat5g137120	Psat5g147600	Psat5g163320	Psat5g187880		Psat5g210880	Psat5g174880	Psat5g244720
Psat5g139680	Psat5g149520	Psat5g163960	Psat5g193360		Psat5g210920	Psat5g179280	
Psat5g144920	Psat5g149840	Psat5g165320	Psat5g194880		Psat5g215920	Psat5g180400	
Psat5g145760	Psat5g150040	Psat5g170120	Psat5g208960		Psat5g227880	Psat5g199600	
Psat5g148160	Psat5g150080	Psat5g170400	Psat5g209600		Psat5g234040	Psat5g205760	
Psat5g150840	Psat5g152320	Psat5g174760	Psat5g218400		Psat5g243200	Psat5g206920	
Psat5g152600	Psat5g153560	Psat5g177360	Psat5g221360		Psat5g244720	Psat5g216520	
Psat5g153880	Psat5g154040	Psat5g177840	Psat5g242320			Psat5g217320	
Psat5g154600	Psat5g155840	Psat5g182840				Psat5g219840	
Psat5g154920	Psat5g156240	Psat5g189160				Psat5g225480	
Psat5g157440	Psat5g157240	Psat5g189240				Psat5g227160	

Psat5g162040
Psat5g162600
Psat5g163320
Psat5g164000
Psat5g165320
Psat5g165440
Psat5g168480
Psat5g169920
Psat5g174440
Psat5g174480
Psat5g175680
Psat5g176320
Psat5g176720
Psat5g178520
Psat5g179560
Psat5g179600
Psat5g182360
Psat5g185760
Psat5g190720
Psat5g198600
Psat5g205880
Psat5g206120
Psat5g206360
Psat5g212440
Psat5g212640
Psat5g214160
Psat5g219360
Psat5g219400
Psat5g224400
Psat5g224640
Psat5g224680
Psat5g226560
Psat5g227560
Psat5g157800
Psat5g158080
Psat5g158720
Psat5g160600
Psat5g161560
Psat5g163160
Psat5g164040
Psat5g165160
Psat5g167040
Psat5g168640
Psat5g170400
Psat5g171040
Psat5g172400
Psat5g172440
Psat5g177560
Psat5g177960
Psat5g181600
Psat5g183640
Psat5g183760
Psat5g187880
Psat5g189200
Psat5g190640
Psat5g190680
Psat5g198960
Psat5g204800
Psat5g205320
Psat5g206880
Psat5g210000
Psat5g212040
Psat5g212080
Psat5g212560
Psat5g213520
Psat5g215840
Psat5g195320
Psat5g200040
Psat5g205960
Psat5g207080
Psat5g208360
Psat5g213120
Psat5g217800
Psat5g219400
Psat5g227840
Psat5g229680
Psat5g231800
Psat5g237520
Psat5g237840
Psat5g238400
Psat5g239320
Psat5g243560
Psat5g244400
Psat5g245240
Psat5g230840

Psat5g237320	Psat5g221440	
Psat5g237640	Psat5g222320	
Psat5g239440	Psat5g223360	
Psat5g240400	Psat5g224080	
	Psat5g224120	
	Psat5g225840	
	Psat5g226840	
	Psat5g231240	
	Psat5g233920	
	Psat5g234120	
	Psat5g234160	
	Psat5g240640	
	Psat5g240920	
	Psat5g243800	
	Psat5g244080	
	Psat5g244160	
	Psat5g244840	

2012