GENOME-WIDE ASSOCIATION STUDY OF HEAT TOLERANCE IN RAPESEED/CANOLA (*BRASSICA NAPUS* L.)

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Genome-wide association study of heat stress tolerance in rapeseed/canola was conducted in greenhouse, growth chamber, and in the field. A total of 37,539 SNP markers were used in this study. In the greenhouse and growth chamber study, 5, 8, and 7 QTL were found associated with pollen sterility, sterile/aborted pods, and number of pods on main raceme, which explained 46.3%, 60.5% and 60.6% phenotypic variations, respectively. In the field study, 6, 11, 7, 11 and 7 QTL were identified causing phenotypic variation of 52.2%, 71.8%, 53.2%, 73.5% and 61.0% for plant height, main raceme height, pods on main raceme, pod length, and pod abortion on main raceme, respectively. Three QTL located on chromosome C05 and, five QTL on chromosome A10 and C03 were identified linked to two common traits sterile/aborted pods, and number of pods on main raceme, respectively. Multiple heat tolerant candidate genes were identified surrounding these QTL.

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LIST OF ABBREVIATIONS

QTL…….Quantitative Trait Loci

QTL…….Quantitative Trait Loci

GBS…….Genotyping By Sequencing

SNP…….Single Nucleotide Polymorphisms

LD…….....Linkage Disequilibrium

TASSEL……..Trait Analysis by Association, Evolution and Linkage

GWAS…….Genome-Wide Association Studies

IPCC……Intergovernmental Panel on Climate Change

USDA……..United States Department of Agriculture

DNA…….Deoxyribonucleic acid

FDA……..Food and Drug Administration

GRAS……...Generally Recognized as Safe

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CHAPTER 1. GENERAL INTRODUCTION

The global air temperature is increasing day by day, and is expected to rise up to 1.8–4.0ºC than the current level by 2100 (IPCC, 2007). The increasing temperature is creating an abiotic stress modifying the surrounding niche of the crop plants, creating possibly lethal environments for the growth, development and reproduction of crops. High temperature stress changes the morphological, physiological, biochemical, and molecular properties of plants. The crop growth at flowering stage is highly sensitive to heat stress (Kaushal et al., 2016; Bita et al., 2013) which causes flower abortion, pollen sterility; reduced pod development and seed set, as wee as reduced assimilatory capacity (Wheeler, 2006) and productivity (Barnabas et al., 2008) of crops. It can also change gene expression (Ivashuta et al., 2002; Steward et al., 2002) through genomic rearrangements, demethylation of transposons (Bennetzen, 2000) which affects gene activation or deactivation, the capture of a gene fragment and co-suppression of gene activity. Enzyme activities become less efficient beyond the optimum temperature range (Mahan and Upchurch, 1988) due to their structural change (Bensaude et al., 1990). Certain genotypes are more tolerant to heat stress and these tolerant traits are genotype dependent as well as controlled by multiple genes (Prasad et al., 2006; Challinor et al., 2007) .

Like many other crop species, rapeseed/canola (*Brassica napus* L., 2n = 4x = 38, genome AACC) is also suffered from heat stress. Rapeseed/canola is an amphidiploid species of *Brassicaceae*, which is evolved by the spontaneous interspecific hybridization between two diploid species *B. rapa* (2n = 20, genome AA) and *B. oleracea* (2n = 18, genome CC) (Nagaharu, 1935). It is widely grown for the production of edible oil for human consumption, bio-fuel, and animal and poultry feed as a source of high quality protein. It is predominantly grown as in North America, Europe, Australia, China, India, and Bangladesh. To date, it is the

second largest oil producing crop in the world next to soybean (Foreign Agricultural Service, USDA, October 2015). North Dakota is the largest canola producing states of the United States, which produces around 84% of all U.S. canola that contributes more than $384 million to the national economy (5 years average from 2011-2015; USDA-NASS, 2016). Other states with high canola acrage are Oklahoma, Montana, Idaho, Washington, Minnesota, and Oregon.

Canola is very sensitive to heat stress. Generally, 15-20˚C is suitable for its growth and development. High temperature (over 27˚C) causes pollen sterility and pod abortion (Morrison, 1993; Angadi et al., 2000; Nuttall et al., 1992), whereas between 28ºC to 35ºC causes 54% to 87% seed yield reduction, respectively (Gan et al., 2004). It has been estimated that 1°C temperature increase from the suitable range cause 10% yield reduction (Nuttall et al., 1992). Heat stress during pre-anthesis stage reduced pollen fertility, whereas post anthesis heat decreased female fertility of *Brassica* (Young et al., 2004).

Due to limited geographic range and intensive breeding, especially for zero seed erucic acid and low seed glucosinolate content canola germplasms show a comprehensive linkage disequilibrium, which has led to a comparatively narrow genetic basis in the current breeding material (Hasanuzzaman et al., 2013). Genome-wide association study (GWAS) is a powerful tool to identify the genetic architecture of traits and multiple candidate genes associated with the traits in many crop species (Huang et al., 2012; Li et al., 2013; Li et al., 2014). It is based on the historical recombination events and a genome scanning with high-density DNA markers to locate the genetic loci associated with the traits of interest at a relatively high level of resolution (Nordborg and Weigel, 2008; Huang and Han, 2014). To date, GWAS is widely used technology to identify the association of many phenotypic traits with its genotypes of many crop species such as; rice (*Oryza sativa* L.), maize (*Zea mays* L.), barley (*Hordeum vulgare* L.) (Ersoz et al.,

2009; Ordonez et al., 2010), wheat (*Triticum aestivum* L.) (Gurung et al., 2014), soybean (*Glycine max* L.) (Mamidi et al., 2014), shorgum (*Sorghum bicolor* L. Moench) (Morris et al., 2013), tomato (*Solanum lycopersicum* L.) (Sauvage et al., 2014), bean (*Phaseolus vulgaris* L.) (Cichy et al., 2015) including rapeseed (*Brassica napus* L.) (Li et al., 2016). Germplasm-based study creates an opportunity to assess many alleles simultaneously with creating broader genetic variation and a wider background for marker-traits correlation (Hansen et al., 2001). GWAS of canola using SNP (Li et al., 2014) and wide accessions of germplasms (Hasan et al., 2008) as mapping populations can therefore help to find out significant markers associated with heat stress oriented phenotypic traits and the potential candidate genes that are underlying to control the traits. Identification of highly significant markers within and among wide accessions of *B. napus* germplasms through association mapping could be helpful to develop improved canola varieties efficiently while incorporated into the breeding program for a specific purpose. It will help breeders to create genetic diversity which will foster in developing commercially desired canola varieties with specific phenotypes.

The objectives of this study are

1. To screen heat stress tolerant germplasms from wide accessions of spring type Brassica germplasms
2. To identify genomic regions associated with heat stress using genome-wide association mapping
3. To identify candidate genes located around significant QTL regions associated with tolerance to heat.

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CHAPTER 2. LITERATURE REVIEW

2.1. Evolution of rapeseed (*Brassica napus* L.)

Rapeseed (*Brassica napus* L.) is an allopolyploid, specifically an amphidiploid (2n = 4x = 38, AACC). It is originated by the hybridization of diploid species, *B. rapa* (2n=20, AA) and *B. oleracea* (2n=18, CC) (Fig. 2.1). The hybridization and the relatedness of canola with other *Brassica* species was described by the "Triangle of U" (Nagaharu, 1935; Raymer, 2002). According to the description, three diploid *Brassica* species, *B. oleracea* (CC, 2n=18), *B. nigra* (BB, 2n=16) and *B. rapa* (AA, 2n=20) hybridized in three independent events to produce three amphidiploids, *B. juncea* (AABB, 2n=36), *B. napus* (AACC, 2n=38**),** and *B. carinata* (BBCC, 2n=34). It was speculated that the natural hybridization between *B. rapa* and *B. oleracea* occurred several times, which helps to adapt the *B. napus* species, and now it is one of the most economically important edible oilseed crops in the world with 400 years of domestication (Gomez-Campo and Prakash, 1999). The relationships between cultivated *Brassica* species were first clarified by Moringa (1934) and verified by Nagaharu (1935) (Raymer, 2002).

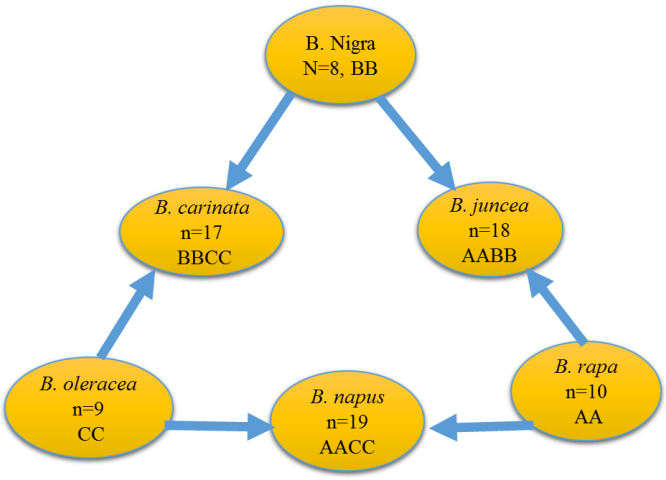

Figure 2.1. "The triangle of U" showing genomic relationships among *Brassica*. Adapted from U 1935 (Nagaharu, 1935).

2.2. Origin, domestication and dissemination of rapeseed (*Brassica napus* L.)

Brassica species have been cultivated for many years and are among the oldest cultivated plants (Raymer, 2002). Archeological records have been dated back to 5000 BCE (Yan Z, 1990; Raymer, 2002) while written records of *Brassica* species have been dated back to ca. 1500 BCE (Prakash, 1980; Raymer, 2002). *Brassica napus* L. (rapeseed) may have arisen in cultivation since no wild species are known (Raymer, 2002; Wang et al., 2011).

Rakow (2004) reported that wild forms of *B. napus* occurred in Sweden, the Netherlands, and Britain. *Brassica* is related to *Arabidopsis* and diverged from a common ancestor about 20 million years ago (Yang et al., 1999; Wang et al., 2011; Koch et al., 2000). *B. napus* arose within the past 10,000 years (Wang et al., 2011). The wild progenitor parental species have been found in the Mediterranean area (Raymer, 2002). During the middle Ages, the oilseed production may have been started in Europe due to the use of oil in lamps. Oilseed rape production is dominated by North America (particularly Canada), Western Europe and China; however, *Brassica* oilseed crops also play a major role in Eastern Europe, the Indian subcontinent and Australia. Since the 18th century, forage rape had been grown in Canada, but the earliest record of rapeseed production in Canada is from 1936 and was credited to a migrant farmer (Mr. Fred Solvoniuk) who moved from Poland to Saskatchewan, Canada (Bell, 1982; Khachatourians and Sumner, 2001). The rapeseed brought from Poland was later identified as *B. rapa* (Polish type).

2.3. Taxonomy of rapeseed (*Brassica napus* L.)

The species *Brassica napus* L. belongs to the genus *Brassica* and the family *Brassicaceae*, which was formerly known as crucifereae. Approximately, 338 genera and 3709 species are belonging to this family (Cheng et al., 2014). The genus *Brassica* consists of around 100 species including *B. napus* L. (Thomas, 2003) which is generally called oilseed rape or

rapeseed. The flowers of this species are bisexual, cruciform petals and composed of four petals, four sepals, a pistil with two carpels and six stamens, the outer stamens are shorter than the rest. Flowers are arranged in a branching type of inflorescence called a raceme. The flower is yellow, the ovary is superior type and positioned above receptacle of the flower (Bilay, 1976). *B. napus* is a self-pollinating crop, but under favorable environmental condition about 12-47% cross-pollination can occur (Becker et al., 1992). Mature and fertile flowers produce a large amount of pollen, and the pollen transfer to the adjacent flowers through insect, wind, and also physical contact which helps pollination of this crop. Fruits are long, slender, and called as pod or silique (Bilay, 1976).

2.4. Rapeseed/ canola

Canola stands for “Canadian Oil Low Acid”, which is a trademark of the Canola Council of Canada. Two species, *B. rapa* and *B. napus*, are commonly known as rapeseed/canola which must meet the following internationally regulated standards: "the oil shall contain less than 2% erucic acid in its fatty acid profile and the seed meal shall contain less than 30 micromoles glucosinolates per gram of air-dry, oil-free meal" (Canola Council of Canada. 2014a). The United States Food and Drug Administration (FDA) granted Generally Recognized as Safe (GRAS) status in 1985 to canola oil (Brown et al., 2008). Canola and rapeseed became the most widely grown non-cereal crops in North America by 1983.

2.5. History of canola

Canola is an important oil producing crops over the course of the past three decades, containing low erucic acid in oil and low glucosinolates in seed meal. During the Second World War, the increasing demand of industrial lubricant leads the necessities of canola research and cultivation in Canada. In 1954, Canada developed a Brassica variety “Golden” (Stefansson,

1983) which contained the higher percentages of erucic acid (C22:1, cis 13-docosenoic acid) and high glucosinolates, both were harmful for human consumption and livestock feed. By the continuous effort of the researchers, in 1963, the scientists of University of Manitoba, Canada, identified low erucic acid containing *B. rapa* line "Liho" (Stefansson, 1983; Downey et al., 1989) and the first Canadian *Brassica* variety with low erucic acid, 'ORO' (*B. napus*), was released in 1968 (Bell 1982; Khachatourians et al., 2001). The value of this crop was not still up to the mark due to the presence of high quantity harmful glucosinolates content in the seed meal. In 1969, the Polish spring rape (*B. napus*) variety 'Bronowski' was identified as a low-glucosinolate content variety. This cultivar was used in a backcrossing program to introgress this polygenic trait into high-yielding erucic acid-free rapeseed variety. Finally, in 1974, Dr. Baldur Stefansson developed for the first time the '00'quality spring oilseed rape variety, 'Tower', (Brown et al., 2008) with low erucic acid in the oil and low glucosinolates in the meal.

2.6. Importance of canola

Canola oil is considered as healthy vegetable oil for human consumption with a healthy fatty acid profile: about 7% saturated, 61% mono-unsaturated, and 32% poly-unsaturated (21% linoleic, 11% alpha-linolenic acid) fatty acid content (http://canolainfo.org/quadrant/media/files/health/canola-oil-good-for-every-body.pdf). The oil is also rich with alpha-linolenic acid (ALA) which has been related to a lower risk of cardiovascular disease (Connor, 2006). Canola oil contains high monounsaturated fatty acid, which is good for heart and control worst cholesterol (LDL) regulating the blood glucose and also increase frying stability of the oil. Due to low erucic acid level in canola, it becomes very healthy and digestible vegetable oil (Campbell, 1963). This oil is mostly used in frying and baking, margarine, salad dressing, preserving of food stuffs etc. Canola oil is also enriched and well balanced with polyunsaturated fatty acids, linoleic acid and

alpha-linolenic acid, and vitamin E (Canola council of Canada, 2013) which are recognized as nutritionally favorable. Moreover, canola meal is also a good source of protein for animal feed (http://www.ers.usda.gov).

2.7. Economic importance of canola in North Dakota

The cultivation of this crop is increasing day by day and to date, it is the 2nd largest source of vegetable oil in the world next to soybean (Foreign Agricultural Service, USDA, 2015). Rapeseed/canola became the most widely grown non-cereal crops in North America by 1983, but in the U.S., canola cultivation was started in 1991. U.S. canola seed production, planting acreage and crop value increased sharply from 1992 to 2015 (USDA-NASS, 2015). The sharp increases from the average of five years between 1992–1996 to the average of five years between 2011-2015 for seed production is 627%, cultivation area is 488% and the crop value is 1,285% (Fig.2.2). Among the United States, the highest cultivation acreage is located in North Dakota state which produces about 84% of U.S. canola (5 years average from 2011-2015, USDA-NASS). Most of the acres of North Dakota are concentrated near the U.S Canadian border. Other states that grow canola include Oklahoma, Montana, Idaho, Washington, Minnesota, Oregon, and the Pacific Northwest. In 2015, the economic value in the top three states in production was $386.26 million (North Dakota), $20.85 million (Oklahoma), and $12.12 million (Montana) (Table 2.1).

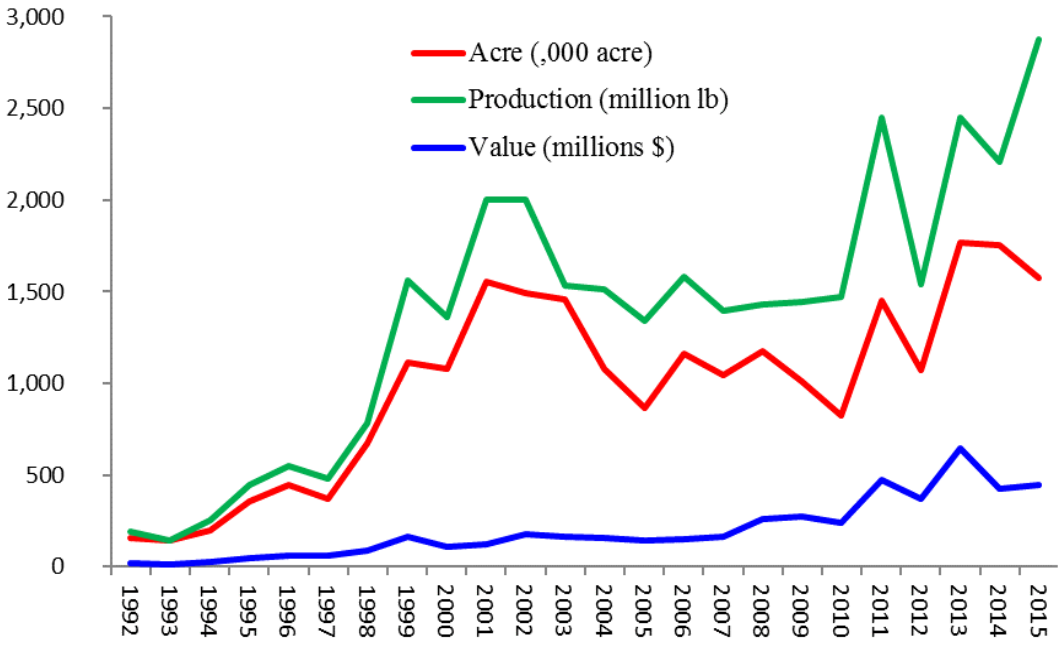

Figure 2.2. U.S. Canola seed production (green), planting area (red) and crop values (blue) since 1992 (USDA-NASS, 2015).

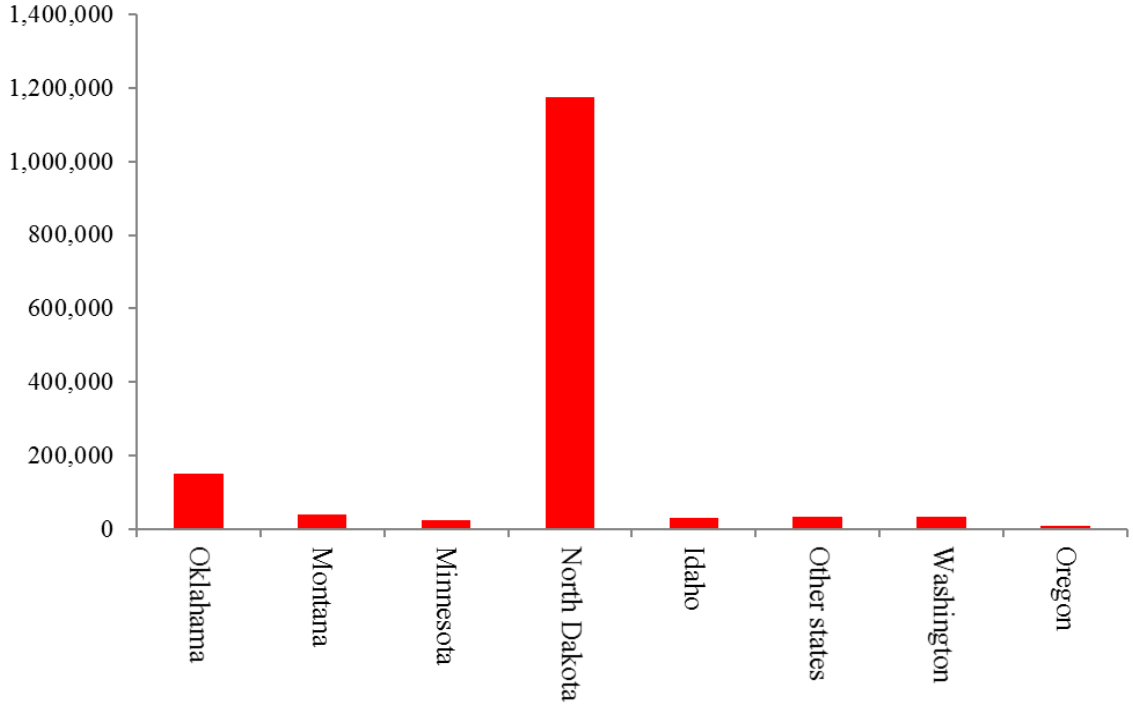

Figure 2.3. U.S. canola plant acreage by major states (5 years average from 2011 to 2015) (USDA-NASS, 2015).

Table 2.1. Canola price per Cwt (Centum weight) and value of production in different states of the United States from 2013 to 2015.

State	Price per Cwt (US $)			Value of production(US $*1000)		
	2013	2014	2015	2013	2014	2015
Idaho	22.0	16.0	16.0	17,501	9,792	6,048
Minnesota	26.4	20.0	18.0	8,494	4,713	7,276
Montana	19.0	16.1	13.4	20,189	13,553	12,124
North Dakota	20.6	17.0	15.5	343,052	361,998	386,260
Oklahoma	20.3	15.0	15.9	42,346	14,415	20,845
Oregon	22.0	18.7	17.5	4,259	2,805	567
Washington	21.5	18.4	17.5	13,158	10,378	6,545
Other states	19.6	17.0	17.5	7,449	5,728	5,849
United states	20.6	17.0	15.5	456,448	423,382	445,514

Source: National Agricultural Statistic Service (NASS), USDA. Crop values 2015 summary (February 2016). (http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1050)

2.8. Molecular characterization of *Brassica* species

Rapeseed/Canola (*Brassica napus* L., AACC) has 19 chromosomes, 10 are from its progenitor species *B. rapa* (AA) and 9 are from the other progenitor species *B. oleracea* (CC). The genome size is about 1,130 Mb (Chalhoub et al., 2014). The C genome is larger than the A genome and this is consistent to the genome sizes of *B. oleracea* and *B. rapa*. Transposable elements (TEs) compose only about 34.8% of the genomes. About 101,040 gene models have been estimated using various methods, including RNA sequencing, *Ab initio* gene prediction, protein and EST alignments, and transposon masking. Confirmed matches composed about 91,167 gene models between the genomes of *B. oleracea* and *B. rapa* (Chalhoub et al., 2014). Comparison between orthologous gene pairs of *B. oleracea* (C) and *B. rapa* (A) suggested a divergence time of about 7500 to 12,500 years ago while the *B. napus* would have formed after this divergence date (Chalhoub et al., 2014).

2.9. Heat stress and its effect on crop production

The Globe is warming day by day. It is expected that the global air temperature will be rising up to 1.8–4.0ºC than the current level by 2100 (IPCC, 2007), and it will be detrimental for the crop production in the near future. The increasing high temperature will create an abiotic stress by modifying the surrounding niche of living organisms including crop plants. High temperature may create lethal environments for growth and development of plants from which they must be able to escape. Plants try to cope with the stress by reducing the growth and development as well as the yield, but under lethal condition they fail to survive and ultimately die. High-temperature stress produces different types of metabolites, toxins in plants and alters the hormonal activity which leads plants to show some abnormal behavior under the stressed condition. The increasing high temperature plays a vital role in plant growth, development, physiological process and reduces the production of crops as an abiotic stress (Fig. 2.4). Heat stress reduces the assimilatory capacity (Wheeler, 2007) and productivity (Barnabas et al., 2008) of crops through reducing photosynthesis (Zhang and Zhou, 2006), radiation use efficiency (Hasanuzzaman et al., 2013) and increasing leaf abscission and senescence, shoot and root growth inhibition or fruit damage (Vollenweider and Günthardt-Goerg, 2005), increasing respiration (Reynolds et al., 2007), higher production of Reactive Oxygen Species (ROS) (Guo et al., 2007), lipid peroxidation, and protein degradation in various metabolic processes (Savchenko et al., 2002).

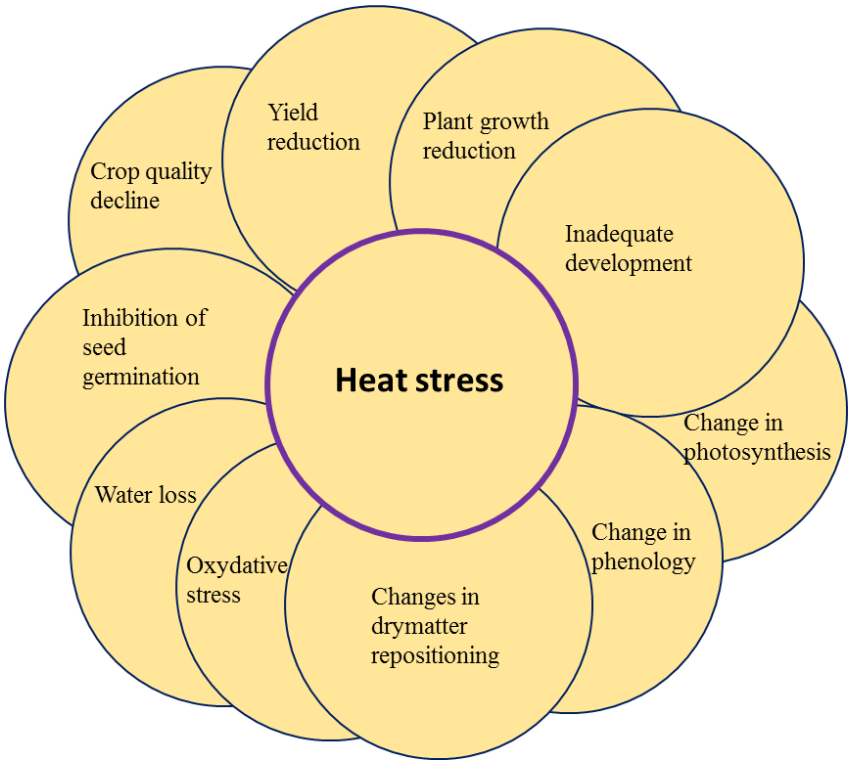

Figure 2.4. Effect of heat stress on plant growth, development and physiological process (adapted from Hasanuzzaman et al., 2013).

Heat stress induces pollen sterility and reduce the yield in many crop species including canola (Zinn et al., 2010). Many essential phytohormones like ABA, salicylic acid (SA) and ethylene (ET) are increased under heat stress and the other phytohormones like cytokinin (CK), auxin (AUX), and gibberellic acids (GA) are decreased and the effects of these hormonal change ultimately cause premature plant senescence (Talanova et al., 2003; Larkindale and Huang, 2004; Larkindale et al., 2005). High temperature plays a significant role in reducing plant growth by affecting the shoot net assimilation rates and reducing the total dry weight of plant (Wahid et al., 2007). High temperature disrupts biosynthesis and compartmentalization of metabolites in plants (Maestri et al., 2002) and it also change carbon metabolism activities, accumulation of carbohydrates and synthesis of sucrose by regulating specific genes (Ruan et al., 2010). It also changes the gene expression which ultimately cause tapetum degeneration and pollen sterility in

several plant species that affects ultimate yield and quality of plant species (Oshino et al., 2007; Endo et al., 2009).

2.10. Effect of heat stress on rapeseed/canola

Increasing high temperature has a significant impact in the *Brassica* growing regions of the world. High temperature stress might cause severe seed yield reduction and therefore plant breeders have been working to develop heat tolerance germplasm (Malcolm et al., 2002). Canola is a cool season crop, of the 15-20ºC are suitable for its growth and development. This crop is highly sensitive to high temperature stress (Morrison, 1993; Brandt and McGregor, 1997; Angadi et al., 2000). High temperature creates an abiotic stress for the growth, development, and reproduction of canola which can cause substantial yield losses of this species (Angadi et al., 2000; Morrison and Stewart, 2002). Plants suffer at different level of heat stress at their different growth stage, particularly prior to fertilization the effect is more severe. Seed yield potentiality of this crop is depends on the stress occurrence and stress severity prior to and during the flowering stage (Mendham, N. J. and Salisbury, 1995), while the crop gets heat stress during the reproductive period, the significant yield loss occurs in *Brassica* (Hall, 1992). Heat stress significantly affect pollen development, anthesis and the fertilization stage of *Brassica* (Hall, 1992) while the photosynthetic source is also indirectly affected by high temperature stress (Morrison, 1993; Angadi et al., 2000; Craufurd et al., 2003). Morrison and Stewart (2002) reported that high temperature at 29.5ºC from bolting to the end flowering stage significantly reduced the seed yield of three different *Brassica* species: *B. napus, B. rapa* and *B. juncea*. Shorter periods of heat stress are also found destructive during the critical growth stage of *Brassica* species. Angadi et al. (2000) conducted an artificial heat stress simulating study during flowering stage on three species of *Brassica* under controlled conditions with 35/15ºC day/night

temperature for 7 d and identified an 89% seed yield reduction of the main stems, and a 52% seed yield decreased per plant. Seed storage components, including oil and proteins are generally accumulating between 2 and 5 weeks after flowering in oilseed rape. In an another heat stress experiment on three *B. juncea* cultivars and one *B. napus* cultivar with a heat stress of 35/18ºC (day/night) for 10 days, the seed yield per plant was reduced by 77% at the pod development stage, 58% at the flowering stage, and 15% at the bud formation stage (Gan et al., 2004). A moderate heat treatment of 28/23ºC (day/night) for 10 d on *B. napus* from 20 to 30 days after flowering altered the fatty acid profile including increased oleic acid and reduced linoleic and linolenic acids of rapeseed oil. A very high temperature heat stress of 38/23ºC (day/night) for 5 d from 25 to 29 days after flowering reduced the seed oil content and the seed yield (Aksouh et al., 2001). Heat stress during bolting to the end of flowering stage on *B. juncea* and *B. rapa* significantly reduced the seed yield of these two species through inhibiting flower, silique and seed production (Young et al., 2004). Planting time has a great effect on temperature stress. The delay planting of canola gets high temperature stress mostly during anthesis or prior to a pod formation stage, which leads higher rate of pod abortion and lower seed yield (Thurling, 1974; McGregor, 1981). Polowick and Sawhney (1988) conducted an experiment with a canola variety 'Westar' imposing high temperature stress (32/26ºC) in a growth cabinet and reported that heat stress resulted in sterile flowers with smaller sepals, petals, and stamens. Late bud development to early seed development stage of *B. napus* cultivars Westar and Delta were found more sensitive to heat stress at 27/17ºC (day/night) with almost sterile flower or pods (Morrison, 1993).

2.11. Heat stress tolerance

Heat stress tolerance is a multigenic character. Many biochemical and metabolic pathways are involved with heat stress. Plants alter its morphological, physiological and genetic architecture to cope with the increasing heat stress. There are several traits like, antioxidant activity, membrane lipid unsaturation, gene expression and protein translation, protein stability, and accumulation of different compatible solutes are associated with the development and maintenance of high temperature tolerance of plants (Kaya et al., 2001). High temperature stress produces a huge amount of heat shock protein (Vierling, 1991) that act as thermotolerance ingredient and molecular chaperones to prevent denaturation or aggregation of target proteins in plant cell (Lohar and Peat, 1998; Ahuja et al., 2010; Scharf et al., 2012). As soon as the plants get heat stress stimuli in the plasma membrane of the cell, it stimulates the messengers such as Ca2+ ion, calmodulins (Liu et al., 2003) and calcium-dependent protein kinases (CDPKs) (Das and Pandey, 2010) that act as calcium sensor, which ultimately activate a novel class of protein called Heat Shock Proteins (HSPs). These HSPs serve as molecular chaperons to maintain protein functions as well as cellular protein refolding, thereby protecting plants under heat stress conditions (Wang et al., 2004). High temperature affects the gene through transcriptional repression of genes, DNA polymerases and deregulation, methylation of DNA, which are involved in cell growth of plants (Sakata and Higashitani, 2008; Smith and Workman, 2012).

Plants alter their metabolic process, maintain cell turgor pressure, arrange proteins, cellular structures and alter the antioxidant system to recreate redox balance and homeostasis in cells against high temperature to become tolerant to heat stress (Valliyodan and Nguyen, 2006). The high carbohydrate content of tomato plants demonstrated heat tolerance through increasing the sink strength and sugar signaling activities under extreme heat stress (Firon et al., 2006).

Plants express genes to protect from the heat stress through changing osmoprotectants, transporters, detoxifying enzymes, and regulatory proteins (Semenov and Halford, 2009) which makes it tolerant against the stress. Scientists are still working to develop heat stress tolerance in plants. However, the active heat stress tolerance in plants through induction of genes is still to be unknown (Frank et al., 2009).

In *Arabidopsis thaliana*, heat stress transcription factors (HSFs) regulate many heat responsive genes with heat shock proteins (HSPs) those are responsible for thermotolerance during the vegetative growth stage of *Arabidopsis* (Scharf et al., 2012). Yang et al. (2006) reported that 0.25 to 12 h heat stress treatment of 7-d–old seedlings of *B. rapa* (cv. Jangwon) significantly increased the accumulation of HSFs and HSPs and also up-regulated some cell wall modifying genes against the heat stress. MicroRNAs (miRNAs) also play a significant role in heat stress tolerance (Yu et al., 2012). Developing gametophyte of *B. napus* showed responsive to heat stress while at least one HSP transcript was found prominent in pollen and pistil during the reproductive stage of this crop, which suggests that the developing gametophytes are also responsive to high temperature stress (Young et al., 2004). Giorno et al. (2013) reported that the heat stress during pollen development stage increases the gene expression as well as HSFs and HSPs for male reproductive organs while the cell proliferating gene and, genes involved with DNA replication and genes encoding hydrolytic enzymes in tapetum cells, were silenced to protect the developing pollen from excessive heat stress.

Seeds are the harvested organs of *B. napus* and heat stress causes significant loss of oil and starch of seed during seed filling stage. The short-term solution against heat stress and development of heat stress tolerance is quietly unknown to the scientists due to the complexity of physiological traits of crops and their interaction with the surrounding environmental conditions

(Shao et al., 2007). Recently, crop simulation modeling, combined with the genetic information of crop plants is becoming an additional methodology to identify the complex physiological traits of plants (Semenov and Halford, 2009), which might be helpful to identify heat stress related complex traits as well as to develop heat stress tolerance of crops.

2.12. Single nucleotide polymorphism (SNP) markers in *Brassica napus*

Markers are the highly heritable genetic "tags" that helps to identify the genes associated with a specific phenotypic expression which are difficult to identify phenotypically. Markers can also be used to map genes that controls the traits of interest (Thoday et al., 1961). Among different molecular markers, SNP markers are comparatively new, stable, simple to use for genotyping. SNPs are currently one of the most popular markers for the fine mapping of heritable traits (Chagné et al., 2008). In many species, these markers are distributed throughout the genome, which are frequently used in the genome-wide association mapping study (Drenkard et al., 2000). SNPs can also be applied to genetic diagnostics, germplasm identification and marker-assisted selection for breeding programs in agriculture. SNPs are also excellent genetic markers for high-density genetic map construction, physical ordering of chromosome, QTL mapping, association mapping and linkage disequilibrium (LD) studies, and also for the comparative and evolutionary genomic analyses. There are three categories of SNPs: transversions (C/G, A/T, C/A and T/G), transitions (C/T or G/A) and insertions/deletions which is also known as indels. SNPs are the frequently used marker which are contributing the majority of genotyping work in different crop species including *B. napus* (Durstewitz et al., 2010; Trick et al., 2009; Westermeier et al., 2009).

The availability of reference genome of *Brassica napus* (Chalhoub et al., 2014) make it feasible to genome-wide identification of SNPs in many allopolyploid *Brassica napus*

accessions. Genotyping by sequencing (GBS) is a low costing technology based on SNPs which is basically involved in low coverage of a large group of samples or large scale genotyping. SNPs are used for the analysis of genetic diversity in *Brassica*, where the high heritability of SNPs is an excellent indicator of genetic diversity and phylogeny in *B. napus*. These markers are also used for association studies because of their availability, low rate of reversion to their ancestral state, and comparatively low cost of high throughput assay. SNP mapping is a helpful tool to know the validation of current *B. napus* genome sequencing and the effort of its assembly and evolutionary process. Trick et al. (2009) reported around 20,000 SNPs across the genome of *B. napus* mapping population BnaTNDH to identify the parents of the population. According to Duran et al. (2010), with the availability of the 50K Infinium SNP chip for *B. napus*, it will be helpful to map the larger size of the polyploid genome of different crop species.

Different studies have also shown that *B. napus* has an SNP of every 600 base pairs of the genome (Edwards et al, 2007; Fourmann et al., 2002) which indicate that ~ 1.1 Gb size of the *B. napus* genome would have equate to ~ 1.7 million SNP markers. This distribution of SNPs in *Brassica* genome help to identify many important genetic traits associated with phenotypic expression of *B. napus* (Duran et al., 2010; Edwards and Batley, 2004; Edwards, 2007). In many studies, SNPs were used to identify the major QTL in canola particularly for its oil yield, oil quality, disease resistance and pod shatter tolerance etc. (Rahman et al., 2008; Kaur et al., 2009; Pilet et al., 1998 Qiu et al., 2006; Smooker et al., 2011) which were found helpful to construct a high density QTL mapp of this crop species. Choi et al. (2007) described that SNPs areenablede to fine-mapping of the QTLs. Hayward et al. (2012) stated that the discovery of genes required for resistance to the major fungal pathogen *Leptosphaeria maculans* (the causal

agent of blackleg disease) with the help of SNPs associated with the genes are the candidates for qualitative or quantitative trait nucleotides (QTNs) which also called the perfect markers.

Genotyping based on the identification of SNPs in its complex allotetraploid genome is an important criteria of *B. napus* for association mapping. Among many high-throughput genotyping assays, Amplifluor (Serological Corp), the Affymetrix Genechips, the SNPlex, TaqMan and SnaPshot assays are used in applied Biosystems for quality genotyping (Appleby et al., 2009). Now a day, Golden Gate and Infinium based genotyping is widely used as high-throughput technology for the genotyping assays with SNPs (Fan et al., 2006). Most recently, the Infinium® II assay is introduced as a custom chip for *B. napus* which has been designed for screening around 50,000 genome-specific SNPs simultaneously.

2.13. Association mapping

Association mapping (AM), also known as linkage disequilibrium mapping, is a relatively new and promising genetic method for complex trait dissection of plants. It is one of the most important tools to study the complex traits of plants with linkage disequilibrium (Zhu et al., 2008) and it is also a method of QTL mapping through the linking of phenotypes to genotypes (Yu et al., 2006). AM helps to develop a high resolution map, and greater allele frequency (Yu and Buckler, 2006). AM uses a sample of accessions from the germplasm collections which are found from the many rounds of meiosis and hereditary recombinations within the ancestors of the samples or population. The main objective of AM studies is the detection of correlations between genotypes and phenotypes in a genetically diverse populations/ individuals/ genotype core collection based on linkage disequilibrium (Zondervan and Cardon, 2004). There are two types of AM, (1) candidate-gene association mapping which is associated with polymorphisms in selected candidate genes controlling the phenotypic variation for

particular traits, and (2) genome-wide association mapping (GWAS) which is associated with genetic variation within the whole genome to find signals of association for various complex traits (Risch and Merikangas, 1996). AM is a relatively low cost technology that can also be used for abiotic stress related traits in combination with marker assisted selection breeding (Varshney et al., 2009). This is also useful to conduct the experiment repeatedly and this approach will help to detect the appearance of alleles in different germplasms where allele specific markers can be used for the introgression of the genes into the commercially cultivated varieties. Lamkey et al. 2013. reported that AM is able to identify coherent marker-QTL associations across populations. Although AM is a widely used and most popular approach to map linkage disequilibrium, in some cases there is a probability of getting false positives due to a strong population structure, and also in some cases high linkage disequilibrium can cause poor resolution of the map. Therefore, population structure, genotyping and accurate phenotyping are the pre-requisite for the high resolution association mapping (Balding, 2006). The first association mapping study was conducted for human diseases identification where genome-wide association study (GWAS) gave a good result to diagnose major loci associated with type 2 diabetes disease (WTCCC, 2007). In *Brassica* research, the first application of the association mapping approach was used to identify the marker-trait association for seed quality traits in *B. napus* core collection (Lühs et al, 2003a). This approach was also used to see the association between plant height and primary branch of *B. napus* (Li et al., 2016), genetic architecture of seed weight and quality in rapeseed (Li et al., 2014), genetic architecture of flowering time (Xu et al., 2015), blackleg disease of rapeseed (Rahman et al., 2016), clubroot disease of rapeseed/canola (Zhang et al., 2016) etc. In plant genetics, AM was first applied to Oat genomic study in 1998 to identify the significant

QTL using restriction fragment length polymorphisms (RFLP) markers where 13 significant QTL was identified from 64 oat varieties (Beer et al., 1997; Virk et al., 1996).

The first step of AM is the selection of germplasms, cultivars, or breeding lines with a wide range of genetic diversity. Phenotyping of the selected population is the next step of this approach. Then it needs to accumulate genotypic data of the germplasms with molecular marker information. Markers which possess less than 5% minor allele frequency (MAF) should be removed from the marker groups to avoid the lower resolution of the association within the alleles (Myles et al., 2009). Linkage disequilibrium determination, assessment of the population structure and kinship, development or selection of the regression model is the next step of AM. The better model is selected on the basis of smallest mean square difference (MSD) between the observed and expected P-value. General linear model (GLM) and Mixed linear model (MLM) are used to control the population structure, where GLM is used to control only fixed effects, but MLM to control both fixed (SNP and population structure effect) and random (kinship) effects (Yu et al., 2006). As MLM deals with unbalanced data across multiple trials and shows reliable inference through the correlation of model between genetic and environmental effects, so it is used in GWAS to avoid biasness within the population structure and relatedness. The molecular markers present within the close proximity of traits of interest are known as significant markers and used as a "marker tags" in this approach (Abdurakhmonov et al., 2008). Significant markers (based on *P*-value) could be subsequently used in stepwise regression model to identify major QTL. Finally, markers from each side of the major QTL will be blasted to identify candidate genes.

2.14. Importance of phenotyping in association mapping

Precise phenotyping is one of the important criteria to get a high resolution map in association mapping. Collection of high-quality phenotypic data is the part of the accurate phenotyping and uses of these high quality phenotypic data increase the precision of AM. Although the cost of genotyping is decreasing rapidly, but the demand of efficient phenotyping is increasing than the increasing number of SNPs to increase the power in association studies. The association mapping study is based on the phenotypic data of the diverse population, but the phenotypic scoring and the accuracy is costly and time consuming. Besides, for the precise data, it needs replicated trial in different environments to reduce the environmental effects and the reduction of errors on phenotyping. Data collected from different environments shows diversity, but, the mean phenotype data from different environments shows the precise data without the environmental effects. Flint-Garcia et al. (2005) reported that the newly discovered candidate genes in association mapping studies can only be validated if we have accurate and robust replicated data from different environments and different years. To ensure the high quality phenotypic data from a different experiments and different environments, it is necessary to monitor the performance and environmental growth conditions (field or greenhouse) which should be included as an annotation to the experiment in the trait database. Therefore, to increase the power of association mapping and to get a robust map, it is necessary to consider efficient field designs, appropriate statistical methods, and QTL × environmental interaction (Eskridge, 2003).

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CHAPTER 3. GENOME-WIDE ASSOCIATION STUDY OF TOLERANCE TO HIGH TEMPERATURE STRESS OF CANOLA IN CONTROLLED CONDITION

3.1. Abstract

High temperature plays a significant role in growth, development and yield of *Brassica napus*. Even a short period of heat stress can lead to 15-20% of yield loss of this crop. A total of 88 accessions were studied to identify the effect of short periods high temperature stress on the early flowering stage of *Brassica napus*. Two sets of accessions with three replications per set were grown in a greenhouse at 22/18°C day/night temperatures. Plants from set-2 at 6-day flowering stage were exposed for a short period to an artificial heat stress simulating conditions in a plant growth chamber. The heat stressed plants were recovered at 22/18°C day/night temperatures in a greenhouse. Data on pollen sterility, sterile/aborted pods, and number of pods on main raceme were taken from both control (set-1) and heat stress (set-2) plants. The heat susceptibility index for each trait was calculated and an association mapping study was conducted using 37,539 SNPs to identify the genomic region controlling the heat stress. A total of 115 and 15 significant markers were identified associated with the heat tolerant traits using P=0.01 and P=0.001 cutoff with 10000 bootstraps, respectively. With stepwise regression, a total of 5, 8, and 7 QTL were identified associated with pollen sterility, sterile/aborted pods, and number of pods on main raceme, which together explained 46.3%, 60.5%, and 60.6% phenotypic variations, respectively. Many candidate genes were identified associated with the QTL related to male sterility, pollen abortion, embryo abortion, reduce plant growth etc.

Keywords: *Brassica napus,* heat stress, controlled environment, QTL, association mapping.

3.2. Introduction

Rapeseed/canola (*Brassica napus* L, AACC, 2n=4x=38) is an amphidiploid species of *Brassica*, originated from two diploid species *B. rapa* (2n=20, AA) and *B. olerecea* (2n=18, CC) (U, 1935). It is the second largest oil producing crop in the world after soybean (Foreign Agricultural Service, USDA; October, 2015). This crop is cultivated as a major oilseed crop in Canada, Europe, China, Australia, USA and the Indian subcontinent. North Dakota is the largest canola producing states with around 84% of U.S. canola production that contributing about $384 million to the national economy (5 years average from 2011-2015; USDA-NASS, 2016).

It has been predicted from climate models that the global mean temperature will increase by 1–4°C by the end of the twenty-first century (Driedonks et al., 2015). The increasing temperature will create an adverse environment that will impact agriculture and crop production (IPCC, 2007). This abiotic stress changes morphological, physiological, biochemical, and molecular properties of plants. The crop growth at flowering stage is highly sensitive to heat stress (Kaushal et al., 2016; Bita et al., 2013), and causes flower abortion, pollen sterility, reduced pod development, seed set, assimilatory capacity and productivity of crops (Wheeler, 2007; Barnab et al., 2008). Certain genotypes are more tolerant to heat stress and these tolerant traits are genotype dependent as well as controlled by multiple genes (Prasad et al., 2006; Challinor et al., 2007).

Rapeseed/canola is highly affected by heat stress. Generally, 15-20°C temperature is suitable for growth and development of canola. High temperature (over 27°C) causes pollen sterility, pod abortion and significant yield loss of this crop (Morrison, 1993; Angadi et al., 1999; Nuttal et al., 1992). It has been estimated that every 1°C temperature increase from the suitable range of its growth and development during pod setting can cause 10% yield reduction of canola

(Nuttal et al., 1992). Heat stress during pre-anthesis stage reduces the pollen fertility, whereas post-anthesis heat decreases the female fertility of *B. juncea* (Rao et al., 1992).

Heat stress tolerance in plants is a complex phenomenon, where numerous biochemical and metabolic activities such as antioxidant activity, membrane lipid unsaturation, gene expression and translation, protein stability, and accumulation of compatible solutes are involved (Kaya et al., 2001). This is a polygenic trait that makes it difficult to introgress multiple favorable alleles into cultivars (Frova and Gorla, 1993; Ottaviano et al., 1991).

Canola germplasms show a comprehensive linkage disequilibrium due to its limited geographic range and intensive breeding (Hasanuzzaman et al., 2013). Genome-wide association study (GWAS) is a powerful tool to identify the genetic architecture of traits and multiple candidate genes associated with the traits in many crop species (Huang et al., 2012; Li et al., 2013; Li et al., 2014). It is based on the historical recombination events and a genome scanning with high-density DNA markers to locate the genetic loci associated with the traits of interest at a relatively high level of resolution (Nordborg and Weigel, 2008; Huang and Han, 2014). GWAS is widely used technology to identify the association of many phenotypic traits with its genotypes of many crop species. This study helps to find out significant markers associated with different qualitative and quantitative traits through using diversified populations.

Genome wide association mapping using the spring type diversity germplasm panel will help to uncover multiple small effects of quantitative trait loci (QTL). In the light of this, an association mapping study was performed to pinpoint genomic regions controlling the heat stress tolerant traits.

3.3. Materials and methods

3.3.1. Plant materials and phenotyping

A total of 122 spring type *B. napus* accessions obtained from USDA-ARS Germplasm Resources Information Network were used in this study. The accessions were grown in a greenhouse and plant growth chamber of North Dakota State University (NDSU), Fargo, USA during 2014-2015. Two sets of experiments (set-1 and set-2) were conducted in randomized complete block design with three replications per set. The set-1 experiment (control) was conducted in the greenhouse at 22/18° day/night temperature until desiccation. The germplasm in the set-2 were grown in the same greenhouse, and a short periods of heat stress was given at 6-day old flowering plants in the plant growth chamber for 5 days. The artificial heat stress simulating condition was set up as 18ºC for 4 hours, temperature ramped up from 18ºC to 35ºC in 6 hours, maintained at 35ºC for 4 hours, and temperature ramped down from 35ºC to 18ºC in 6 hours. The relative humidity in the growth chamber was maintained at 70%, and light was provided for 14/10 hours day and night. The plants in the growth chamber were watered twice a day with a rate of 300 ml/application/plant. After the heat treatment, the plants were returned into the original greenhouse room at 22/18° day/night temperature and grown until desiccation. The flowering buds were tagged before and after heat stress to identify the pod development during the period of heat stress. Flower buds were collected from the heat stressed plants as soon as plants were removed from the growth chamber after 5 days of heat stress, while buds from the controlled plants were collected at the same time. Data on pollen sterility, sterile/aborted pods on main raceme, and number of pods on main raceme were taken from all the plants grown in set-1 (control) and set-2 (heat stress treatment). The pods that have already been aborted/ sterile without any seed are counted from the main raceme as sterile/ aborted pods. On the other hand,

total number of pods were counted on the main raceme with viable and sterile pods. Although we had planted 122 germplasms for the study, however due to late flowering, as well as lack of GBS data, finally we were able to use a complete data of 88 germplasms (Table A8, S9, & S10) in association mapping study. ANOVA was performed through SAS Proc GLM procedure using SAS 9.3 (SAS Institute, Inc. 2011) (Freund et al., 1986) to see the significant variation among the genotypes (Table A2, S3 and S4). The standard deviation, skewness, kutosis, and normality (Kolmogorov-Smirnov test) was calculated (Table A1 & Suplementary Fig. S1). Pearson correlation was also performed using R 3.3.0. to see relationhip among the traits (Table A5).

Figure 3.1. Heat simulation study under normal condition in the greenhouse (left) and heat stressed condition in growth chamber (right).

3.3.2. Pollen sterility study

The pollen sterility study was conducted in the laboratory. The flower buds prior to open were collected from the greenhouse, and growth chamber. A total of 10 buds from each germplasm per replication were collected in an Eppendorf tube containing water, and were stored in an icebox during bud collection. The water from the Eppendorf tube was replaced carefully by

30% acetic acid solution (70% ethanol + 30% Acetic acid) and was preserved in the refrigerator for the study. The preserved buds were open carefully with forceps on the pre-cleaned VWR micro slides (25 mm X 75 mm, and 1.00 mm thick) and 2-3 anther were macerated and pressed using scalpels on each slide with 1-2 drop of 1% acetocarmin solution. The slide was heated over a spirit lamp for 5-10 seconds to fix the acetocarmin dye with the pollen grain. The anther debris was cleaned with needles and a coverslip was placed on the slide carefully to prevent entering air bubble between the slide and coverslip. The sterile (no/lightly stained) and viable (stained) pollen were counted under an optical microscope (N-400M, 110-115V ~ 60/60HZ; 0.4A, Halogen lamp 60V 20W). One hundred random pollen grains per slide were counted and percentage of fertile and sterile pollen grains were recorded. The images of the representative samples of fertile and sterile pollen were captured for future record (Fig. 3.2).

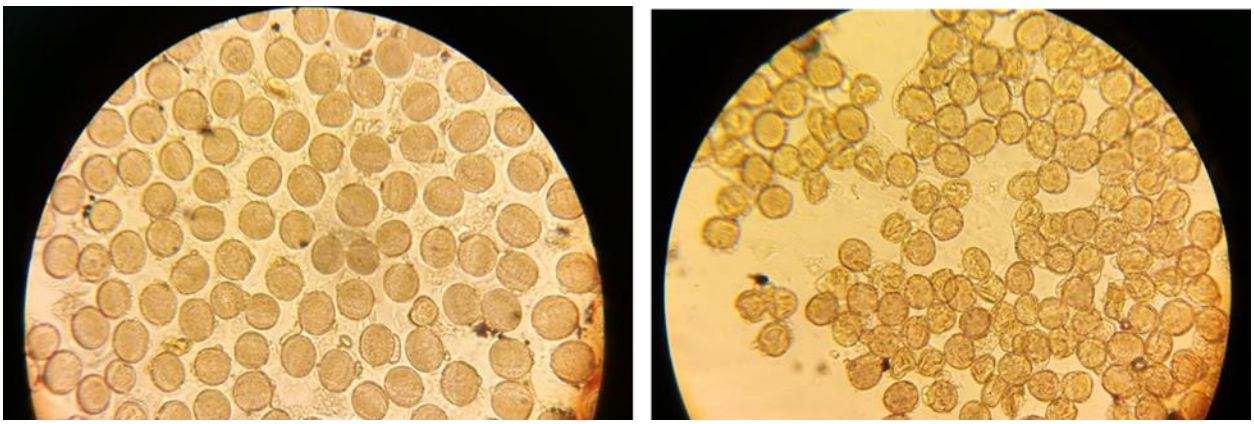

Figure 3.2. Pollen sterility status without heat stress (left- Almost no sterility) and heat stressed condition (right- almost all are sterile).

3.3.3 Heat susceptibility index (HSI)

Heat stress of each trait was calculated as a heat susceptibility index (HSI) using the equation by Fisher and Maurer (1978):

$$\text{HSI} = \frac{(1-\frac{Y_h}{Y})}{(1-\frac{X_h}{X})}$$

where, Y_h and Y are the phenotypic means for each genotype under heat stress and controlled conditions, respectively, and X_h and X are the phenotypic means for all lines under heat stress and controlled conditions, respectively.

3.3.4. DNA extraction and genotyping

DNA was extracted using a Qiagen DNeasy kit (Qiagen, CA, US). The genotyping was conducted at the institute of genome diversity (IGD), Cornell University for genotype-by-sequencing (GBS). The 88 genotypes used in the study are a subset of a core collection of the *B. napus* lines used for diversity study (Michelak et al., Unpublished). The GBS data was cleaned followed by alignment using BWA-MEM. It is a new alignment algorithm which is used to align sequence reads or assembly contigs of a large large reference genome. This algorithm is an automatic process of choosing alignments between local and end-to-end of the alignments. It also supports paired-end reads and align chimeric regions. This algorithm is a robust method of finding sequencing errors and also useful to alignment for small to large sequences (~70 bp to few megabasepairs). To date, this algorithm is more feasible and frequently used method to align 100bp sequences (Li et al., 2013). SNP calling was performed using VarScan. It is a platform-independent software tool which is used to detect variants in NGS data. This software employs a robust statistic approach which is used to call read depth, base quality, variant allele frequency,

and statistical significance of the variants. (Liu et al., 2013). The SNPs were further imputed to estimate the missing alleles using FastPHASE 1.3 (Scheet et al., 2006) to increase the power of the study and mapping the causal variant. All markers <5% MAF were removed for further analysis. The 37,539 SNP marker data used in this analysis for the 88 individuals was cleaned from minor allele frequency (MAF).

3.3.5. Population structure and relatedness

Population structure was analyzed using principal components (PC), estimated in TASSEL 5.0 (Bradbury et al., 2007). PCs that account for 25% (PC_3) and 50% (PC_{17}) of cumulative variations were included in the association mapping analysis. In addition, a pairwise kinship coefficient matrix (K-matrix) was estimated (Fig.3.9) as the proportion of shared alleles for all pairwise comparisons within the population (Zhao et al., 2007). Further an identity by state matrix was also estimated to see the relatedness among the genotypes. The Linkage Disequilibrium (LD) between markers was estimated using TASSEL version 5.0 as the squared allele frequencies correlations (r^2). The LD decay graph was plotted with physical distance (kb) between pairs of polymorphic SNP markers and the correlations of allele frequency ($r^2 = 0.2$) in R version 3.3.0 (Fig. 3.3). The expected decay helps to estimate the numbers of markers needed to scan the whole genome.

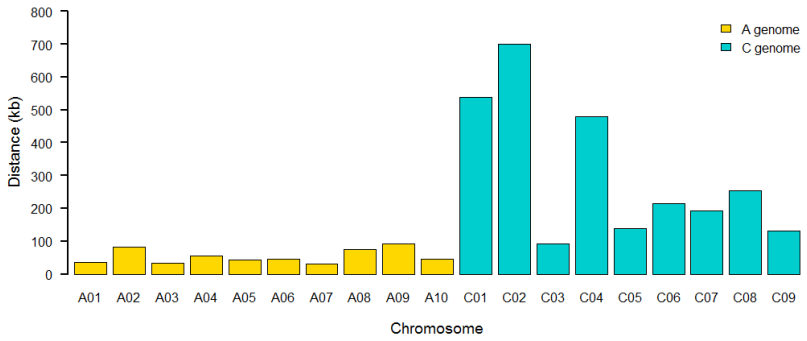

Figure 3.3. Bar graph showing patterns of linkage disequilibrium (LD) decay across 19 chromosomes of canola. Each bar represents the expected rate at which LD decays with physical distance (kb) for a chromosome at a threshold of $r^2 = 0.2$, based on a non-linear regression model.

3.3.6. Genome-wide association analysis

Six regression models (Naïve, PC_3 (25% variation), PC_{17} (50% variation), Kinship, PC_3+Kinship, and PC_{17}+Kinship) were used to estimate *p*-values for each of the markers in TASSEL 5.0 (Bradbury et al., 2007) using general liner model (GLM) and mixed linesr model (MLM). Among the six models for each trait, the best model was selected based on the smallest Mean Square Difference (MSD) between the observed and expected *P*-values (Mamidi et al., 2011). After that, the best model was run against the individual trait using generalized least squares (GLS) model (mixed model) in TASSEL(https://bitbucket.org/tasseladmin/tassel-5-source/wiki/User Manual/MLM/MLM) to identify the phenotypic variation (r^2) by individual markers.

A marker is considered significant if the *P*-value of the marker is within 0.1 and 0.001 percentile tail of 10,000 bootstraps (Mamidi et al., 2014; Gurung et al., 2014). Bootstrapping is the re-sampling of the individual marker trait association. This approach is similar to choose an arbitrary value based on choosing predefined percentile tail from an empirical distribution. As *P*-value is dependent upon phenotypic distribution, the variation explained by the marker, structure

and relatedness of the population so, we had used a cutoff to identify the marker trait association as well as significant markers. In addition, stepwise regression was implemented using significant markers in the SAS REG procedure to estimate the combined variation (r^2) explained by these significant markers as well as to reduce the number of significant markers to define major QTL (Mamidi et al., 2014; Gurung et al., 2014). A significant *P*-value of 0.05 was necessary for both marker and model for stepwise inclusion of the marker in REG procedure in SAS 9.3. Further, genes 100 kb on either side of Major QTL were used to identify candidate genes. The gene sequences of canola were blasted against the *Arabidopsis* gene models (TAIR10 database; Berardini et al., 2015) to obtain an annotation for the gene models. The genes were further subject to a literature search to find the function of the genes related to the traits of interest.

3.4. Results

3.4.1. Phenotypes

Variations between control and heat stress study was observed for all the traits when the plants were grown in normal greenhouse conditions and were exposed to 5-day heat stress during flowering time in the plant growth chamber (Table 3.1). The heat stress treatment significantly increased pollen sterility, sterile/aborted pods, and reduced the number of pods on main raceme. The percentage of pollen sterility and sterile/aborted pods were increased sharply after the heat stress, which were 84.4 times and 26.1 times, respectively. On the other hand, the total number of pods on main racemes were reduced to 0.86 times of the heat treated plants (Table 3.1). The phenotypic estimate is presented in Table A1-S4; Fig. 3.4-3.6.

Table 3.1. Variation within the three traits studied under heat stress and controlled conditions.

Traits	**Set-1 (Control)**			**Set-2 (Heat stress)**			**"X" Change**
	Av.*	Stdev*	Range	Av.*	Stdev*	Range	
Pollen sterility	0.25	0.44	0-2.33	21.1	21.07	0-94.3	84.4
Sterile/aborted pods	0.51	0.96	0-5.67	13.3	6.92	2-36.3	26.1
Pods on main raceme	34.8	11.7	13-74.5	30	10.29	8-66.5	0.86

* Av=Average; Stdev= Standard deviation; "X" Change = Times of phenotypic change.

Figure 3.4. Same germplasms at before and after heat stress (Brown tag- before heat stress and white tag-after heat stress).

Figure 3.5. Status of an NDSU line before and after heat stress.

Figure 3.6. Pollen sterility of a germplasm Legend at before and after heat stress A) GHSE; B) after heat stress.

3.4.2. Genotyping and association mapping

A total 42,575 SNPs were obtained from the samples used in this study. After filtering, a total 37,539 SNPs with minor allele frequencies >5% were further used for the subsequent analysis. There was a total of 20.6% heterozygous loci among these samples. Population structure was analyzed and controlled using principal components (PC) analysis. Three and 17 PCs accounted for cumulative variation of 25% and 50%, respectively, and were used for controlling population structure in the mixed model. Three continuous cluster groups were obtained with the first two principal components through PC analysis (Fig. 3.7). Among the six models used in the analysis, the model with PC_3 (explain 25% variance) and kinship was the best model for pollen sterility, only PC_{17} (explain 50% variance) was the best for the sterile/aborted pod, and kinship was the best model for the total pods on main raceme. The *P-P* plots showed the distribution of observed *P*-values and expected *P*-values for all the three different traits [Fig. 3.8(A, B & C)].

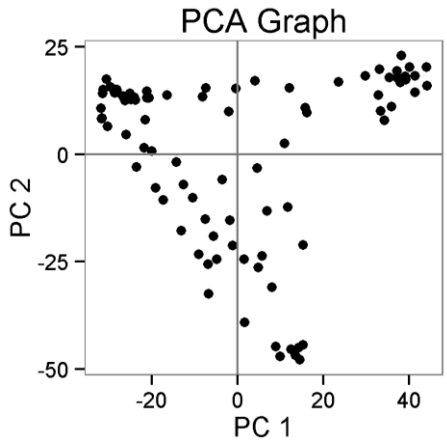

Figure 3.7. PCA graph showing distribution of two principal components of 37,269 SNPs. PC1 and PC2 explain 13.42% and 9.5% variations, respectively.

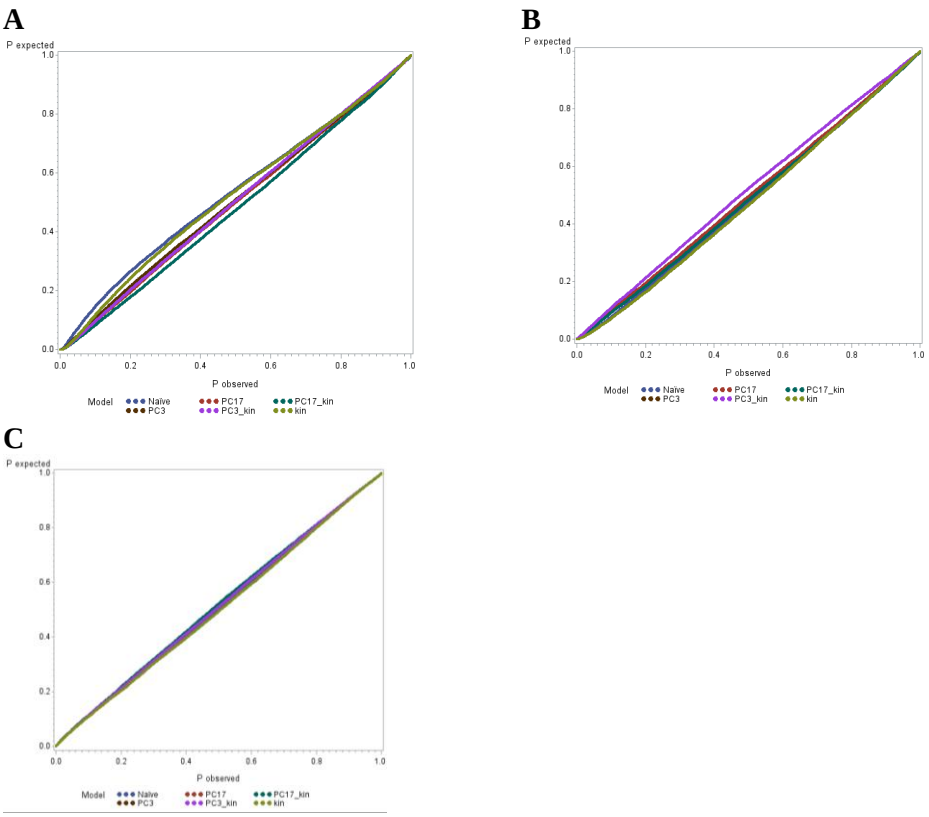

Figure 3.8. (A-C). *P-P* plot: Distribution of *P*-values for the six models tested in relation to three different traits (A) Pollen sterility(%), (B) sterile/aborted pods, (C) total pods on main raceme. *P*-observed value is plotted on the X axis and *P*-expected value is plotted on the Y axis. The different color represents the different regression models used. The best model is that one which is close to the diagonal line.

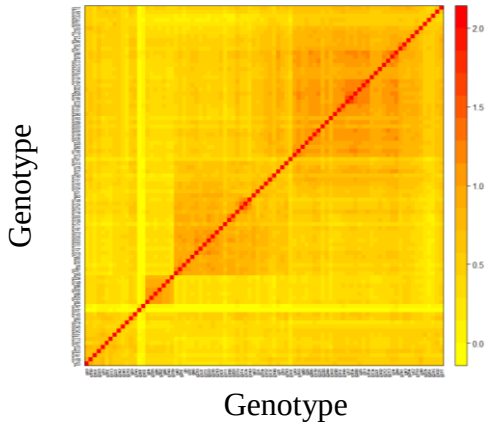

Figure 3.9. Heat map of pairwise kinship among 88 canola genotypes used for controlled study in the greenhouse. The red squares in the diagonal indicate a genotype's genetic relatedness to itself.

3.4.3. Pollen sterility

Six markers were found significant at the 0.001 percentile (p≤6. 38E-08; Table 3.2; Fig. 3.10A), and were located on chromosomes C08 (3.76 Mbp), C03 (0.39 Mbp) and C04 (47.2 Mbp). Two other markers, named chrAnn_rand_4645588, and chrCnn_rand_18549112, were present at unknown positions and evenly distributed on the chromosomes. Another 33 markers were found significant at 0.01 percentile tail of the empirical distribution (p≤5.86E-06; Table A6). These significant markers were found on multiple chromosomes. A stepwise regression was performed to identify the major QTL associated with these markers and five significant QTL regions were identified those explained 46.31% of the total variation (Table 3.3). The candidate genes identified here include genes associated with male sterility, pollen tube growth, pollen abortion and anther dehiscence (Table A7).

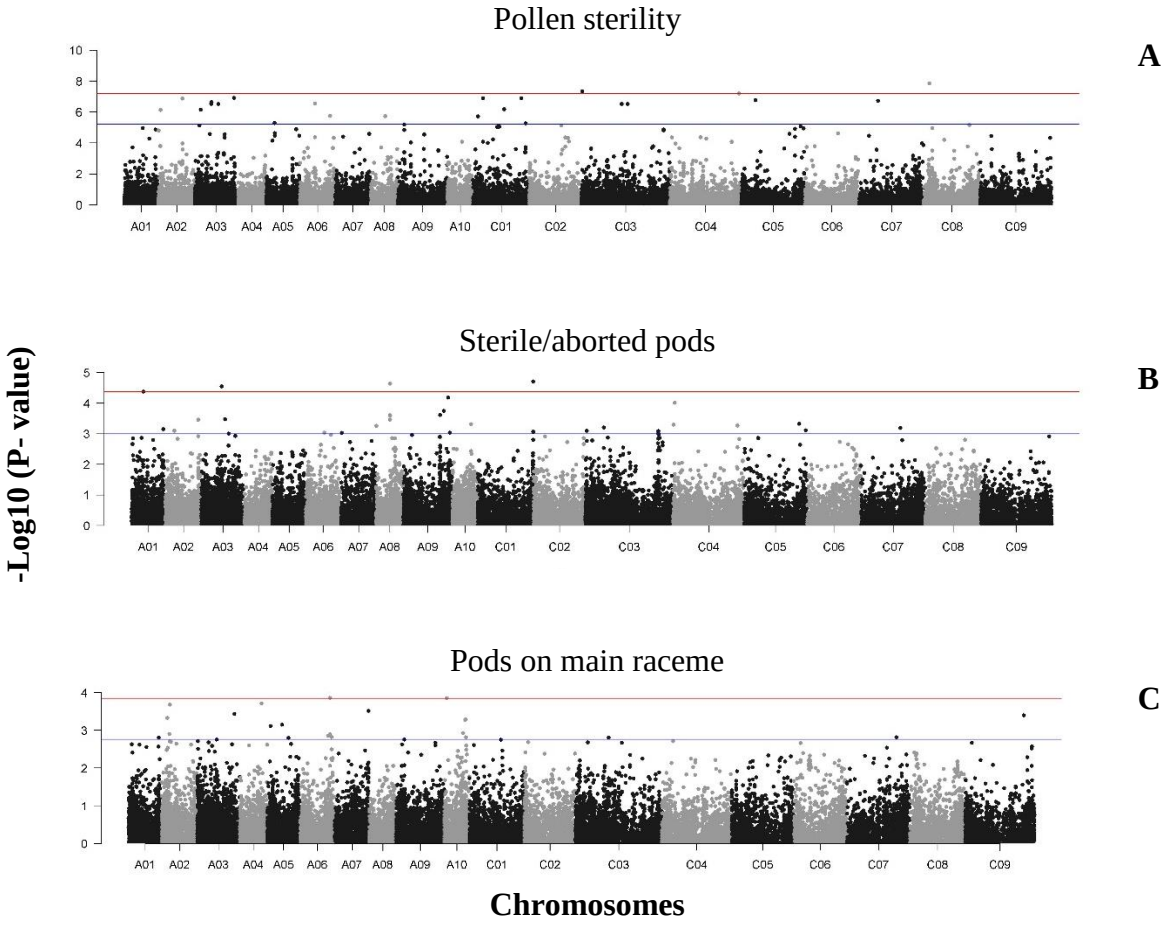

Figure 3.10. Manhattan plots for the three major traits of *B. napus* associated with heat stress. (A) pollen sterility, (B) sterile/aborted pods, and (C) pods on main raceme. Y-axis showing the *P*-value on a -$\log_{10}$ scale and, X-axis showing the nineteen chromosomes (A01-A10 and, C01-09). Horizontal line with red color showing the significant markers with $p \leq 0.01$.

Table 3.2. Significant markers associated with different traits tolerance to heat stress.

Marker	Chr*	Position (Mbp)	*P*-value	-Log_{10} *p*	A1	Mean frequency	A2	Mean	Mean Difference
Pollen Sterility									
chrAnn_rand_4645588	Ann_rand	4.64	1.35E-09	8.87	CC	86.43	TT	1084.1	997.67
chrC03_394217	C03	0.39	4.64E-08	7.33	AA	1084.1	GG	116.81	967.29
chrC04_47184437	C04	47.1	6.38E-08	7.2	CC	1084.1	TT	84.54	999.56
chrC04_47184438	C04	47.1	6.38E-08	7.2	AA	1084.1	TT	84.54	999.56
chrC08_3759455	C08	3.75	1.42E-08	7.85	GG	38.73	TT	1084.1	1045.37
chrCnn_rand_18549112	Cnn_rand	18.5	7.78E-11	10.11	CC	1084.1	TT	86.98	997.12
Sterile/aborted pods									
chrA01_8090850	A01	0.81	4.16E-05	4.38	AA	326.92	GG	234.11	92.81
chrA03_13865121	A03	13.8	2.82E-05	4.55	CC	457.8	TT	217.59	240.21
chrA08_9665233	A08	9.66	2.27E-05	4.64	CC	380.48	TT	253.83	126.64
chrC01_38231402	C01	38.2	1.94E-05	4.71	AA	240.56	GG	392.74	152.18
Pods on main raceme									
chrA02_3316315	A02	3.31	5.47E-05	4.26	AA	1.88	TT	3.64	1.77
chrA06_20870206	A06	20.8	9.02E-05	4.04	CC	3.25	TT	1.88	1.37
chrA06_20955765	A06	20.9	1.37E-04	3.86	GG	3.1	TT	1.97	1.14
chrA10_1645036	A10	1.64	1.39E-04	3.86	GG	2.46	TT	2.33	0.13
chrCnn_rand_35427743	Cnn_rand	35.4	4.88E-05	4.31	AA	1.88	CC	3.64	1.77

*Chr.: chromosome; position (Mbp) the position of the significant markers on chromosome; *P*-value is the Bonferroni *P*- value of SNPs; A1 and A2 represents Allele-1 and Allele-2.

Table 3.3. Significant Markers associated with QTL expressing cumulative phenotypic variation of different traits of canola under heat stress condition.

Traits	# of significant markers	# of QTL	Chromosomes	Position (Mbp)	% Phenotypic Variation
Pollen sterility	39	5	A02	1.5	46.31
			C01	21.3	
			C02_rand	1.4	
			C05	9.6	
			Unn_rand	6.7	
Sterile/aborted pods	39	8	A01	8.1	60.45
			A03	16.3	
			A09	32.1	
			A10	12.8	
			C05	37.9	
			C05	42.4	
			C07	27.3	
			Unn_rand	6.7	
Pods on main raceme	37	7	A02	5.6	60.59
			A05	15	
			A06	21	
			A10	1.6	
			A10	14.4	
			C03	23.3	
			Cnn_rand	8.7	

3.4.4. Sterile/aborted pods

For pod sterility, four markers were identified significant at 0.001 percentile ($p \leq 4.16E-05$; Table 3.2; Fig 3.10 B). These markers were distributed on chromosomes C1 (38.2 Mbp), A8 (9.67 Mbp), A03 (13.9 Mbp) and A01 (8.1 Mbp). Another 35 markers were found significant at 0.01 percentile tail of the empirical distribution ($p \leq 9.9E-04$) (Table A6). Stepwise regression identified eight QTL region, which explained 60.5% phenotypic variation of pod sterility (Table 3.3). These QTL were located on chromosome A01, A03, A09, A10, C05 and C07. Multiple

candidate genes involved in pod abortion, floral organ development, pollen tube growth, embryo and pollen abortion were identified in these QTL regions (Table A7).

3.4.5. Pods on main raceme

Five markers significant at 0.001 percentile ($p \leq 1.39E-04$; Table 3.2; Fig. 3.10C) were identified for total number of pods on main raceme. These significant markers were located on chromosomes A02 (3.32 Mbp), A06 (20.87 and 20.95 Mbp), and A10 (1.65 Mbp). One markers named chrCnn_rand_35427743 was present at unknown positions. Other 34 markers were found significant at 0.01 percentile tail of the empirical distribution ($p \leq 1.73E-03$) (Table A6). Seven QTL regions were identified through stepwise regression. These seven QTL explained 79.65% phenotypic variation and located on chromosome A02, A05, A06, A10 and C03 (Table 3.3). Candidate genes in this QTL include genes involved in the floral organ development, abortion of various organs during development, reduced flowering fertility etc. (Table A7).

3.5. Discussion

Improvement of several traits, including heat stress tolerance is an important criterion to expand the cultivation of this crop in the United States beyond the North Central states as well as different parts of the world. Heat stress negatively affects plant's developmental and physiological processes, reproduction and adaptation (Hall, 2001). The identification of markers, genes, and QTL associated with heat stress related traits during flowering to pod development stages can help to select stress tolerant genotypes for a breeding program. The spring type germplasm accessions used in this study are originated/obtained from 14 countries of 3 continents. This provides a useful resource of genetic diversity for genome-wide association mapping study.

In this study, we simulated an artificial heat stress conditions in a controlled plant growth chamber. This simulation allowed us to reliably control the temperature, humidity, light, moisture in the growth chamber to facilitate the artificial heat stress. None of the above conditions are possible to control under field testing conditions. Moreover, we evaluated the same germplasm in the greenhouse without heat stress, which gave us an ample opportunity to compare the same germplasm between normal growing and heat stress conditions to find out the heat susceptibility index. We have given a 4h/day heat stress at 35°C for 5 days in a plant growth chamber. This artificial heat stress research showed a similar agreement with many researchers such as Angadi et al. (2000) conducted an artificial heat stress simulating study during flowering stage on three species of *Brassica* under controlled conditions with 35/15ºC day/night temperature for 7 d and identified an 89% seed yield reduction on the main stems, and a 52% seed yield reduction per plant. In an another heat stress study on three *B. juncea* cultivars and one *B. napus* cultivar with a heat stress of 35/18ºC (day/night) for 10 days, the seed yield per plant was reduced by 77% at the pod development stage, 58% at the flowering stage, and 15% at the bud formation stage (Gan et al., 2004). A very high temperature heat stress of 38/23ºC (day/night) for 5 d from 25 to 29 days after flowering reduced the seed oil content and the seed yield (Aksouh et al., 2001). Heat stress at 35ºC during bolting to the end of flowering stage on *B. juncea* and *B. rapa* significantly reduced the seed yield of these two species through inhibiting flower, silique and seed production (Young et al., 2004). Polowick and Sawhney (1988) conducted an experiment with a canola variety 'Westar' imposing high temperature stress at 32/26ºC (day/night) in a growth cabinet and reported that heat stress resulted sterile flowers with smaller sepals, petals, and stamens. Late bud development to early seed development stage of *B. napus* cultivars Westar and Delta were found the most sensitive to heat stress at 27/17ºC

(day/night) with almost sterile flower or pods (Morrison, 1993). Morrison and Stewart (2002) reported that high temperature at 29.5ºC from bolting to the end flowering stage significantly reduced the seed yield of three different *Brassica* species: *B. napus*, *B. rapa* and *B. juncea*. The delay planting of canola gets high temperature stress mostly during anthesis or prior to a pod formation stage, which leads higher rate of pod abortion and lower seed yield (Thurling, 1974; McGregor, 1981).

In general, heat stress tolerance study can be conducted at different developmental stages of the plant, however, in this study the heat stress was given to flowering plants at six days after flower initiation. Early flowering stage of the crop is highly sensitive to heat stress and causes the most significant variation of plant's physiological activity (Johanna et al., 2015, Young et al., 2004), and significantly reduces the seed yield (Hedhly et al., 2009; Thakur et al., 2010). It has been reported that the heat stress during flower initiation or pod development of *B. napus* and *B. juncea* is more deleterious than the heat stress during bud initiation (Gan et al., 2004). Heat stress after pod development stage in *Brassica* do not significantly damage the crop yield (Angadi et al., 2004).

We have studied pollen sterility, flower/pod abortion, number of pods on main raceme which were affected by heat stress. This is an agreement with many findings of heat stress induced pollen sterility, flower and pod abortion, and reduced pods per plant in several species including tomato (Levy et al., 1978; Abdul-Baki 1991), *Capsicum annum* L. (Rylski, 1986; Erickson and Markhart, 2002), bean (Konsens et al., 1991), cowpea (Craufurd et al, 1998), pea (Wery and Tardieu, 1997), and cotton (Reddy et al., 1992). In cereals, the pollen tube growth and pollen viability is significantly reduced during the heat stress at reproductive stage (Saini and Aspinall, 1982; Stone, 2001). Heat stress affects the gametophytes and the tapetum layer which

reduces water and nutrient transportation during microspore development (Young et al., 2004). Heat stress also accelerates the early anther development process, progression of pollen mother cell, arrest the cell proliferation and anther cell wall degradation which ultimately cause pollen abortion and male sterility in the flower (Giorno et al., 2013).

Association Mapping is a powerful tool to identify marker-trait associations and genes associated with the trait (Li et al., 2011; Jia et al., 2008). However, the results might be undermined if the false positives are not controlled. To avoid these spurious associations, the marker-trait associations were corrected for population structure and relatedness and a combination of both. Further the significant markers for each trait were selected based on empirical distribution rather than a single *P*-value based on the suggestion by Mamidi et al. (2014) and Gurung et al. (2014). Further to narrow the QTL peaks and finding the markers for marker assisted selection, a stepwise regression was performed (Mamidi et al., 2014). Our analysis identified 20 QTL regions for the 3 traits studied. Even though the traits are physiologically related, we did not find common QTL because of the involvement of multiple physiological pathways into these trait developments. Gene models around these QTL were used to identify the candidate genes. The canola accessions studied here had a lower LD (Michalak et al., Unpublished), and therefore we selected the markers around the 100 kb sequence of each side of the significant QTL. The position of the markers, QTL, candidate genes are based on the canola genome sequence reported by Chalhoub et al. (2014).

We evaluated 88 diversified spring type accessions for association mapping study. Although, it is not a large number of accessions, however, because of diversified origin, we could identify a large number of polymorphic markers (37,539 markers) for trait association study. Therefore, a low number of accessions were used for this association study. Rezaeizad et

al. (2011) conducted an association mapping study using 49 genetically diverse winter type *B. napus* germplasm. Honsdorf et al. (2010) used 84 winter type *B. napus* accessions for association study. Gajardo et al. (2015) used 89 winter type *B. napus* accessions, Jestin et al. (2011) used 128 accessions, and Liu et al. (2016) used 143 accessions. Moreover, we could identify a very high number of SNPs for association mapping study compared to the other studied mentioned above.

Plants respond to increased temperature by changes in biochemical and physiological processes. Cooling and warming temperature alters membrane fluidity and elicit intracellular free-calcium elevation, and is considered the primary event controlling plant responses to temperature (McClung and Davis, 2010). Over the last few years, many molecular-genetic studies demonstrated the effects of temperature on hormone signaling, flowering time, the circadian clock, light-signal transduction, and cold and heat acclimation as the pre-priming of the acquisition of hardiness against heat stress (Penfield, 2008). The variation in these interrelated traits is due to common physiological responses associated with heat stress in plants. Several heat shock proteins (HSPs) act as sensors to heat stress are found within the QTL regions of the traits. These heat shock genes (HSGs) encode HSPs are vital for plant's survival under heat stress conditions (Yang et al., 2006). These proteins are expressed under high temperature and most of these proteins protect intracellular proteins from being denaturation and preserve their stability and function through protein folding, thus it acts as chaperones (Baniwal et al., 2004).

Flowering is one important trait affected by heat stress. Multiple flower related genes like F-box proteins (Jain et al., 2007; Ariizumi et al., 2011), homeobox-leucine zipper protein 17 (Rueda et al., 2005) were identified in the QTL regions. Multiple genes related to floral organs abortion are also affected by heat stress. They include adenosine kinase 2 that hamper flower and

pod development through embryo abortion (Zhang et al., 2014), Cytochrome P450 family that effects the number of siliques and leads to pollen abortion (Bak et al., 2011), MATE efflux family proteins that induce embryo abortion (Zhao et al., 2015). In addition, pentatricopeptide (PPR) repeat-containing family of proteins that lead to embryo abortion (Lurin et al., 2013), the protein kinase family of proteins that are known to induce pollen abortion in Barley (Radchuk et al., 2006) were identified in the QTL regions. Also cytokinin oxidase 7 and indole-3-acetic acid inducible 19, proton pump interactor known to induce flower abortion were also identified (Nico et al., 2015; Song et al., 2015). Genes known to induce embryo and seed abortion like SET-domain containing protein lysine methyltransferase family protein (Pontvianne et al., 2010), Pyruvate kinase family protein (Zhang et al., 2014), Galactosyltransferase family protein (Basu et al., 2015), GDSL proteins (Zhao et al., 2015), phosphatidic acid phosphohydrolase 2 (Eastmond et al., 2010), phosphoenolpyruvate carboxylase 3 (Fischinger et al., 2010) and RGA-like protein 3 (Cheng et al., 2015) were also identified.

Pollen and flower sterility are also affected by heat stress. We identified many candidate genes associated with flower and pollen sterility in our study, which ultimately reduces the yield of this crop. Among different candidate genes, pentatricopeptide repeat (PPR) superfamily protein, which leads to embryo abortion, thus produces the sterile pod (Lurin et al., 2013). The protein 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein increase oxidative stress to reduce flower and pod (Leisner et al., 2014), Coatomer and beta subunit protein involved in the tapetum cell development and fertility restoration (Singh et al.,2015). Protein kinase superfamily creates pollen abortion in barley (Radchuk et al., 2006), whereas peptide transporter5 controls plant cell differentiation and nutrient supply that hamper flower development (Yang et al., 2000). F-box family protein was found responsible to reduce flower

fertility (Ariizumi et al., 2011). In addition, Adenosine kinase 2 protein hampers flower and pod development through embryo abortion (Zhang et al., 2014), glutamine synthetase 1,4 creates oxidative stress and B-deficiency (Bargaz et al., 2015), heat shock factor 3 associated with male sterility (Kim et al., 2001), pectin lyase-like superfamily protein involved in pollen tube growth (Zhao et al., 2015), homeodomain GLABROUS 9 regulates anther dehiscence (Wilson et al., 2011) and Cytochrome P450 was found associated with pollen abortion with reduced number of elongated siliques in *Arabidopsis* (Bak et al., 2011).

The number of pods per plant varied due to the detrimental effects of heat stress. High temperature reduces the pollen fertility, stigma receptivity and also reduces the pollen tube growth, which lead to reduce the pod development in plants. With the physiological and biochemical activities associated with reduced numbers of pods, many genes are also involved in this process. A group of genes associated with a total number of pods was identified through this study such as P450 reductase1 is responsible for pollen abortion with reduced number of elongated siliques in *Arabidopsis* (Bak et al., 2011), Pectin methylesterase31 involved in pollen tube growth (Zhao et al., 2015), basic helix-loop-helix (bHLH) DNA-binding family protein associated with the development and dehiscence of the seed and pod (Hudson et al., 2015). In addition, translocon at the inner envelope membrane of chloroplasts 20 is involved in tapetum function and microspore development in *Brassica* (Dun et al., 2011), 17.6 kDa class II heat shock protein is associated with heat stress tolerant of crop plants (Al-Whaibi, 2010) and K+ efflux antiporter 6 which is involved in pollen tube development and fertilization (Lu et al., 2011).

3.6. Conclusion

The results of our study identified 115 significant markers associated with pollen sterility, sterile/aborted pods, and number of pods on main raceme. After stepwise regression, the markers were reduced from 115 to 15, those are closely associated with major QTL causing maximum phenotypic variation. This result also demonstrates that the use of association mapping was able to identify many QTL regions which were not known before. The candidate genes identified in the QTL regions support the use of these QTL for identification of genotypes with resistance/tolerance to heat stress. Markers identified here could be used for marker assisted selection in plant breeding programs. As this study was conducted only for one year, so, marker validation and 2nd year study could help to recommend the selected markers in marker assisted selection.

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CHAPTER 4. GENOME-WIDE ASSOCIATION MAPPING OF HEAT STRESS TOLERANT TRAITS OF CANOLA (*BRASSICA NAPUS* L.) UNDER FIELD CONDITIONS

4.1. Abstract

Brassica is a cool season crop and is susceptible to high temperatures. Developing heat stress tolerant variety helps the crop to sustain in growing temperatures and to extend the geographical range of cultivation. A total of 85 spring type *Brassica napus* accessions grown in the field with natural heat stress at the end of flowering to the pod initiation stage were phenotyped. An association study was performed to identify QTL associated with heat stress tolerant traits. About 37k markers obtained using genotyping-by-sequencing were used for this study. Multiple markers distributed on most of the chromosomes were identified. Further, a total of 6, 11, 7, 11 and 7 QTL were identified with 52.19%, 71.75%, 53.21%, 73.48% and 61.02% phenotypic variation for plant height, main raceme height, pods per main raceme, pod length, and pod abortion per main raceme, respectively. Multiple candidate genes known to be involved in stress, abortion of different organs were identified in the vicinity of the QTL.

Keywords: *Brassica napus*, heat stress, QTL, Association mapping.

4.2. Introduction

Rapeseed/canola (*Brassica napus* L., 2n=4x=38) is an allopolyploid species, specifically an amphidiploid consisting of genome AACC and originated from the hybridization of two diploid species *Brassica rapa* (2n=2x=20) and *B. oleracea* (2n=2x=18) (U, 1935; Raymer, 2002). Genome size of this crop is about 1,130 Mb and C genome is larger than the A genome, which is consistent with the genome sizes of *B. oleracea* and *B. rapa* (Chalhoub et al., 2014). Rapeseed ranks the second position in the world as oil producing crop next to soybean (Foreign

Agricultural Service, USDA, 2015). In the USA, about 84% of canola is produced in North Dakota and the market value is about $464 million/year (average of 2011-2015) (Foreign Agricultural Service, USDA, 2015).

Although rapeseed is a valuable oilseed crop, but the production of this crop is hampered due to different biotic and abiotic stresses, such as disease, pest, heat stress, drought and cold stress etc. High temperature creates lethal environments for the growth and development of plants, and produces different types of metabolites, toxins and alters the hormonal activity which leads plants to show some abnormal behavior under the stressed conditions. Plants are able to cope with the stress conditions by reducing the growth and development, yield and, changing morphological, physiological, biochemical, and molecular properties. Temperature increases at 3-4˚C from its normal range during reproductive stages, even for a short duration, could cause 15-35% yield loss of many crop species (Ortiz et al., 2008). Generally, the suitable temperature for spring canola production is about 15-20˚C, but the temperature over the 27˚C causes pollen sterility and pod abortion (Morrison,1993; Angadi et al., 2000; Nuttall et al., 1992). The changes of heat stress from 28˚C to 35˚C in rapeseed could reduce the seed yield from 54% to 87%, respectively (Gan et al., 2004). It has been estimated that 1˚C temperature increase from the suitable range of crop growth and development in July, cause 10% yield reduction of canola in Saskatchewan, Canada (Nuttall et al., 1992). Heat stress during pre-anthesis stage reduced pollen fertility, whereas post anthesis heat decreased the female fertility of *B. juncea* (Rao et al., 1992). The generative stage of crop development is highly sensitive to heat stress (Bita and Gerats, 2013) which causes flower abortion, pollen sterility, reduces pod development and seed set, reduces the assimilatory capacity and productivity (Wheeler, 2007; Barnabas et al., 2008) through reducing photosynthesis (Zhang and Zhou, 2006), radiation use efficiency

(Hasanuzzaman et al., 2013), and increases plant respiration (Reynolds et al., 2007), Reactive Oxygen Species (ROS) production (Gong et al., 1998; Volkov et al., 2006, Guo et al., 2007), lipid peroxidation, protein degradation (Savchenko et al., 2002), shoot and root growth inhibition (Vollenweider and Günthardt-Goerg, 2005), hyperfluidization and disruption of plant cell membranes (Horváth et al., 1998; Sangwan et al., 2002), misfolding, and aggregation of protein (Sharma et al., 2010), metabolic imbalance (Vierling, 1991; Gong et al., 1998; Volkov et al., 2006) and yield reduction (Ahuja et al., 2010; Mittler and Blumwald, 2010). Biosynthesis and compartmentalization of metabolites are disrupted by high temperature (Maestri et al., 2002). Heat stress causes tapetum degeneration and pollen sterility (Oshino et al., 2007, Endo et al., 2009), genomic rearrangements (Ivashuta et al., 2002; Steward et al., 2000), demethylation of transposons (Bennetzen, 2000) by regulating specific genes in the biochemical pathway.

Heat stress tolerance in plants is a multigenic character, where numerous biochemical and metabolic traits like, antioxidant activity, membrane lipid unsaturation, gene expression and translation, protein stability, and accumulation of compatible solutes (Kaya et al., 2001) are involved. Transcriptional repression of genes, DNA polymerases, and deregulation of DNA methylation involved in cell growth (Sakata and Higashitani, 2008; Smith and Workman, 2012) are also caused by heat stress. Many genes are expressed to protect plants from the heat stress through changing osmoprotectants, transporters, detoxifying enzymes, and regulatory proteins which makes it tolerant against the stress (Semenov and Halford, 2009). However, the specific role of the genes in heat stress tolerance is not yet identified in crops (Frank et al., 2009). Due to the complexity of physiological traits and their interaction with the environment the short-term solution for heat stress tolerance is quietly unknown to the scientific community (Shao et al., 2007).

Association mapping (AM) is basically developed on the basis of the linkage disequilibrium concept which utilizes ancestral recombination and natural genetic diversity within a population to quantify the quantitative traits (Geiringer, 1944; Lewontin and Kojima, 1960), where linkage disequilibrium is the non-random co-segregation of alleles at two loci. It is an alternative method to discover genetic factors using biparental crosses that has a higher mapping resolution within a large number of unrelated individuals and can identify common genetic variants which control a common phenotype (Risch, 2000). As heat stress is a complex trait, AM would be a good approach to locate the genomic region associated with the heat stress related phenotypes.

In the light of these facts, this research scheme has been taken to pinpoint the genomic region associated with the heat stress traits in a wide accessions of *B. napus* germplasms under field conditions.

4.3. Materials and methods

4.3.1 Phenotyping

A total of 160 spring type *Brassica* accessions obtained from USDA-ARS Germplasm Resources Information Network were used for this study. Plants were grown during 2014 in the field at Prosper, North Dakota, in a randomized complete block design (RCBD) with 3 replications. The germplasms were planted at May 28, 2014 and during flowering, three plants from each replication were tagged randomly for data collection. During the pod initiation time (1^{st} week to 3^{rd} week of July), the air temperature was about 35ºC (https://ndawn.ndsu. nodak. edu), which created a natural heat stress for about 20 days (Table A15). Data on plant height (cm), raceme height (cm), number of pods on the raceme, pod length (cm) and, % pod abortion on main raceme were recorded on the physiological maturity stage of the crop. Plant height was

measured from the bottom (ground level) to the top of the plant. The main raceme height was measured from the bottom to the top of the main inflorescence. Total number of pods on the main raceme were counted for each plant. Pod length was measured from 10 pods per main raceme from the middle part of the main raceme. Pod abortion was recorded from the main raceme. Although we had planted 160 germplasms but due to late flowering, imbalanced flowering, as well as lack of GBS data, finally we got complete data of 85 germplasms (Table A16, & S17), which were used in association mapping analysis. An ANOVA was also performed through SAS Proc GLM procedure using SAS 9.3 (SAS Institute, Inc. 2011) (Freund et al., 1986) to see the significant variation among the genotypes (Table A11). The standard deviation, skewness, kutosis, and normality (Shaprio-Wilk test) was also calculated (Table 4.1, & Suplementary fig. S2). Pearson correlation was also performed using R 3.3.0 to see relation among the traits (Table A12).

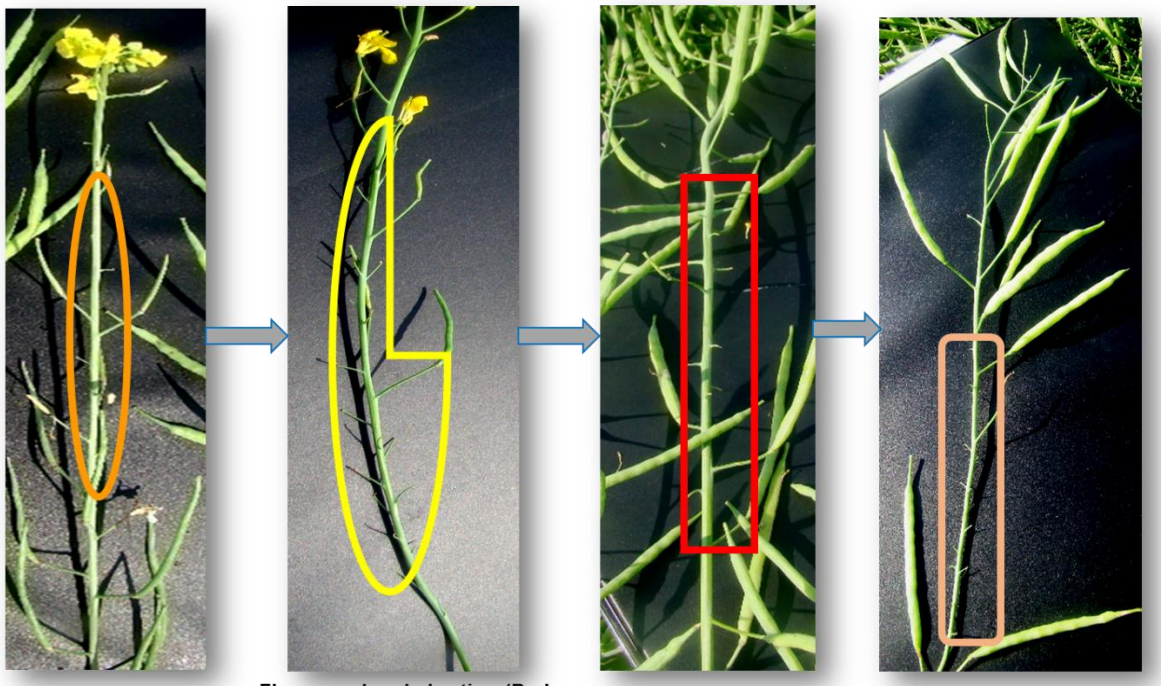


Figure 4.1. Flower and pod abortion under field condition at different stages of pod development.

4.3.2. Genotyping and association mapping

The SNP marker data for these samples were obtained using GBS from a collection of 366 individuals (Michelak et al., Unpublished). Briefly, these samples along with others were digested with *ApkI* enzyme. The Illumina GAII sequencer was used to sequence the sample at 100 bp single end reads. Alignments were performed using BWA-MEM. It is a new alignment algorithm which is used to align sequence reads or assembly contigs of a large large reference genome. This algorithm is an automatic process of choosing alignments between local and end-to-end of the alignments. It also supports paired-end reads and align chimeric regions. This algorithm is a robust method of finding sequencing errors and also useful to alignment for small to large sequences (~70 bp to few megabasepairs). To date, this algorithm is more feasible and frequently used method to align 100bp sequences (Li et al., 2013). SNP calling was performed

using VarScan. It is a platform-independent software tool which is used to detect variants in NGS data. This software employs a robust statistic approach which is used to call read depth, base quality, variant allele frequency, and statistical significance of the variants. (Liu et al., 2013). FastPHASE 1.3 (Scheet et al., 2006) was used to estimate the missing alleles. The marker data for the 85 individuals was further cleaned for minor allele frequency of 5% below which markers were removed. Finally, 37,269 SNPs were subsequently used for the further analysis. We had calculated Linkage Disequilibrium (LD) between the markers as the squared allele frequencies correlations (r^2). This LD was estimated using TASSEL version 5.0. The LD decay graph was plotted with physical distance (kb) between pairs of polymorphic SNP markers and the correlations of allele frequency ($r^2 = 0.2$) using R version 3.3.0 (Fig. 4.2)

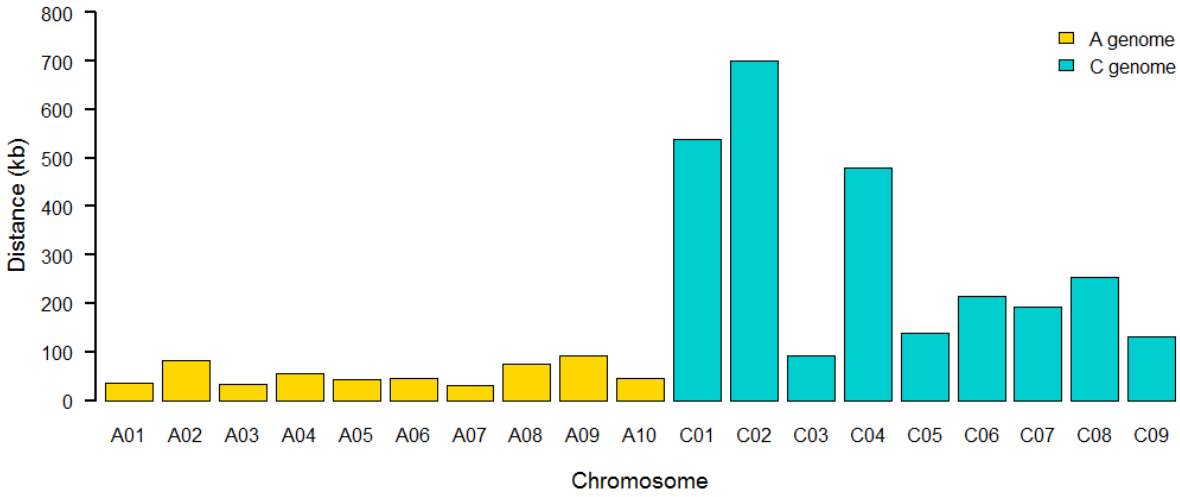


Figure 4.2. Bar graph showing patterns of linkage disequilibrium (LD) decay across 19 chromosomes of canola. Each bar represents the expected rate at which LD decays with physical distance (kb) for a chromosome at a threshold of $r^2 = 0.2$, based on a non-linear regression model.

4.3.3. Structure analysis, kinship, and model testing

Population structure was analyzed using principal components (PC) and estimated in TASSEL 5.0 (Bradbury et al., 2007). PCs that account for 25% and 50% of cumulative variation were used in Association mapping analysis. In addition, a pairwise kinship coefficient matrix (K-matrix) was estimated (Fig. 4.5) as the proportion of shared alleles for all pairwise comparisons within the population (Zhao et al., 2007). Further an identity by state matrix was also estimated to see the relatedness among the genotypes. Six regression models: Naïve, PC_3 (25% variation), PC_{17} (50% variation), kinship, PC_3+kinship, and PC_{17}+ Kinship were used in this study to identify the marker trait association as well as to select the best models. General linear model (GLM) was used for three models (Naïve, PC_3, $PC_{17)}$ as they have only fixed effects, and other three models (kinship, PC_3+kinship, and PC_{17}+ Kinship) were analyzed using mixed linear model (MLM) due to their fixed and random effects. Among the six models for each trait, a best model was selected based on the smallest Mean Square Difference (MSD) between the observed and expected *P*-values (Mamidi et al., 2011) and run the best model using TASSEL to identify the significant markers. The significant markers were identified at p value 0.1 and 0.001 percentile tail of 10,000 bootstraps. Bootstrapping is the re-sampling of the individual marker trait association which is similar to choose an arbitrary value based on choosing predefined percentile tail from an empirical distribution. As *P*-value is dependent upon phenotypic distribution, the variation explained by the marker, structure and relatedness of the population so, we had used a cutoff to identify the marker trait association as well as significant markers.When the *P* -value of a marker is within 0.01 percentile tail of 10,000 bootstraps then the marker was identified as a highly significant marker (Mamidi et al., 2014; Gurung et al., 2014). Significant markers were selected from the selected best models, and Mahhattan plots were constructed

using –log10 of *P*-values against chromosome location for the graphical representation of the position of the markers on chromosome.

4.3.4. Identification of QTL and candidate genes

The best model was run against the individual trait using generalized least squares (GLS) model (mixed model) in TASSEL (https://bitbucket.org/tasseladmin/tassel-5-source/wiki/User Manual/MLM/MLM) to identify the phenotypic variation (r^2) by individual markers.

In addition, stepwise regression was also implemented using significant markers in the SAS REG procedure to estimate the combined variation (r^2) explained by these significant markers as well as to reduce the number of significant markers to define major QTL (Mamidi et al., 2014; Gurung et al., 2014). A significant *P*-value of 0.05 was necessary for both marker and model for stepwise inclusion of the marker in REG procedure of SAS 9.3. Further, genes within 100 kb on either side of the major QTL were used to identify candidate genes. The gene sequences of canola were blasted against the *Arabidopsis* gene models (TAIR10 database; Berardini et al., 2015) to obtain an annotation for the gene models. Candidate genes were identified on the basis of the physiology and functions of those genes which were previously reported.

4.4. Results

4.4.1 Phenotyping of plant materials

Phenotypic variation of the five traits were found variable in the field condition after a short heat stress during flowering to fruit setting stage of the genotypes. Of the genotypes, the number of pods per raceme varied from 13.0 to 52.6 with an average of 30.2 pods per raceme, whereas pod abortion was observed 1.68 to 30.1% with an average of 9.74% in the experiment while the other heat stress related traits like plant height, main raceme height, and pod length

were found different among the genotypes under the study (Table 4.1; Fig. 4.1). Statistics of the phenotypic estimation is presented in Table A11).

Table 4.1. Variation in different traits of *B. napus* under natural heat stress in field condition.

Traits	**Av***	**Stdev***	**Max***	**Min***	**Skewness**	**kurtosis**	**Shaprio-Wilk *P*- value**
Plant height (cm)	96.9	12.6	134	68.0	0.55	1.20	0.0081
Raceme height (cm)	39.9	8.64	61.1	15.5	0.32	0.39	0.1977
# Pod/raceme	30.2	8.15	52.6	13.0	0.5	0.28	0.1503
Pod length (cm)	6.62	0.8	8.21	4.27	-0.76	0.76	0.0055
Abortion%	9.74	5.54	30.1	1.68	1.03	1.32	0.0001

Av* = Average, Stdev*= Standard deviation, Max*= Maximum, Min*= Minimum

4.4.2 Population structure, PCA and relatedness

A total 85 genotypes were used in the gemone-wide association study (GWAS). Polymorphic SNPs were selected on the basis of minor allele frequency distribution. Initially, a total of 42,575 SNP markers were selected, and finally, after removing the minor allele frequency (>5%), 37,269 quality cleaned SNPs were used for the analysis. Principal components (PC) were used to control the population structure. A mixed linear model with 25% and 50% cumulative variation by PCs were used to control the population structure. PC analysis has been grouped the population into three continuous clusters using first two principal components (Fig. 4.3, PC graph).

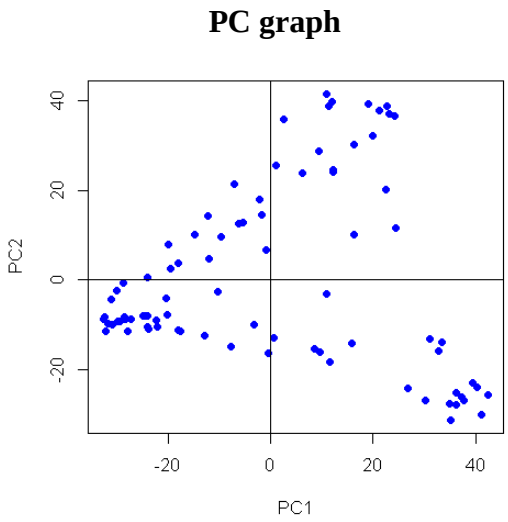


Figure 4.3. PC graph of the first two principal components using 37,269 polymorphic SNPs. The X-axis represents PC1 and Y-axis is PC2. PC1 and PC2 explain 13.13% and 9.5% variations, respectively.

4.4.3. Association mapping (AM)

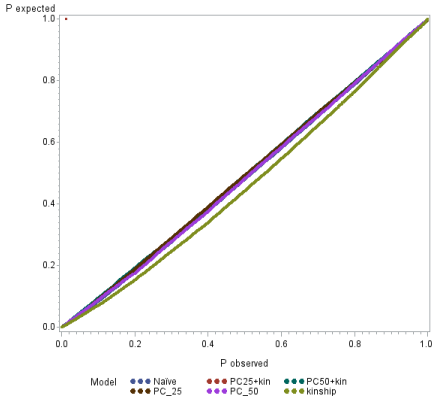
Six regression models were used to test the phenotypic variation associated with the SNPs. Out of the six models tested in the analysis, the model with PC17 (explain 50% variance) and kinship was found as the best model for plant height, and pod abortion. The model PC17 was the best model for the main raceme height and number of pods on main raceme, whereas PC3 (Explain 25% variance) was the best model for pod length (Table 4.2). The *P-P* plots showed the distribution of observed *P*-values and expected *P*-values for all the five different traits [Fig. 4.4 (A-E)].

Table 4.2. Statistics of MSD values of five different traits used in association mapping analysis.

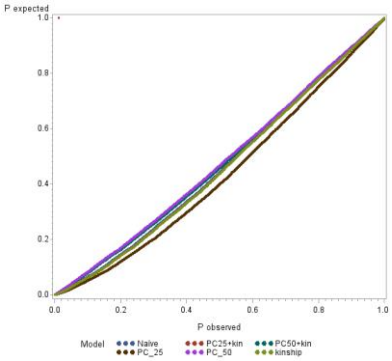
Traits	**Naïve**	**PC_3**	**PC_{17}**	**kinship**	**PC_3+kinship**	**PC_{17}+kinship**
Plant height	0.000264	5.65E-05	0.000239	0.001878	2.179913	**4.5E-05¶**
Raceme height	0.001978	0.005471	**6.59E-04¶**	0.002245	2.179913	0.001031
No of pods on main raceme	0.002282	0.000306	**5.78E-05¶**	0.002247	2.179913	6.29E-05
Pod length	0.000238	**1.43E-04¶**	0.000542	0.000235	2.179913	0.001049
Pod abortion	0.001941	0.000234	0.000323	0.001841	2.179913	**8.73E-05¶**

"¶" is the least mean square difference (MSD) value. PC_3 and PC_{17} are the PCA with 25% and 50% variance, respectively.

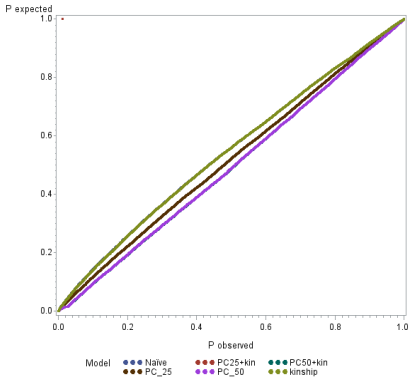
(A) Plant height



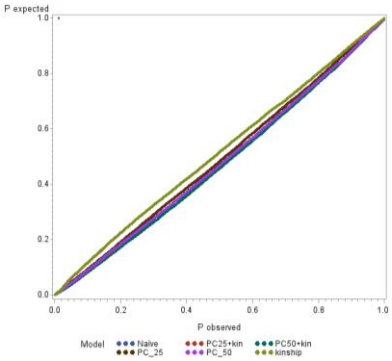
(B) Raceme height



(C) Pods/raceme



(D) Pod length



(E) Pod abortion

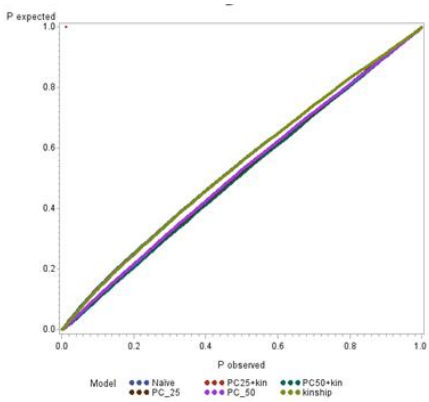


Figure 4.4. (A-E). *P-P* plot: Distribution of *P*-values of six models of the five traits; (A) Plant height, (B) Main raceme height, (C) pods on main raceme, (D) pod length, (E) Pod abortion; Y-axis represents expected *P*-value while X-axis is observed *P*-value.

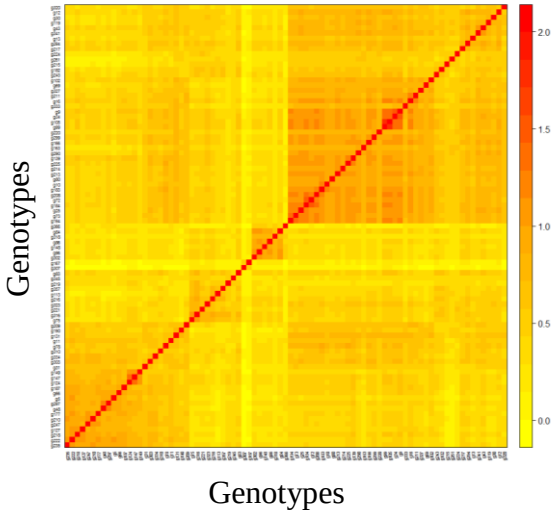


Figure 4.5. Heat map of pairwise kinship among 85 canola genotypes used for field study. The red squares in the diagonal indicate a genotype's genetic relatedness to itself.

During the marker trait association, three markers were found significant for plant height at the 0.001 percentile ($p \leq 2.99E-05$; Table 4.3; Fig. 4.6A) tail of empirical distribution. Among these three markers, two of them were located on the chromosome C03 (0.5 Mbp) and C08 (32.86 Mbp) and, the other one (78.5 Mbp) was randomly distributed in chromosomes without any known position. Another 35 markers were found significant at 0.01 percentile tail of the empirical distribution ($p \leq 5.18E-04$; Table A13). These markers were found on multiple chromosomes. A stepwise regression with these markers identified 6 significant QTL regions associated with this trait that explained 52.19% phenotypic variation (Table 4.4). The candidate genes identified here were: kinase family protein that plays an important role in plant growth and development, iron regulated 2 protein associated with Iron (Fe) availability for plants which is an essential mineral element for plant growth and development, ethylene-responsive nuclear protein (ERT2), regulates plant growth and development through cell division, and gibberellin 2-oxidase that was found involved in plant growth and development (Table A14).

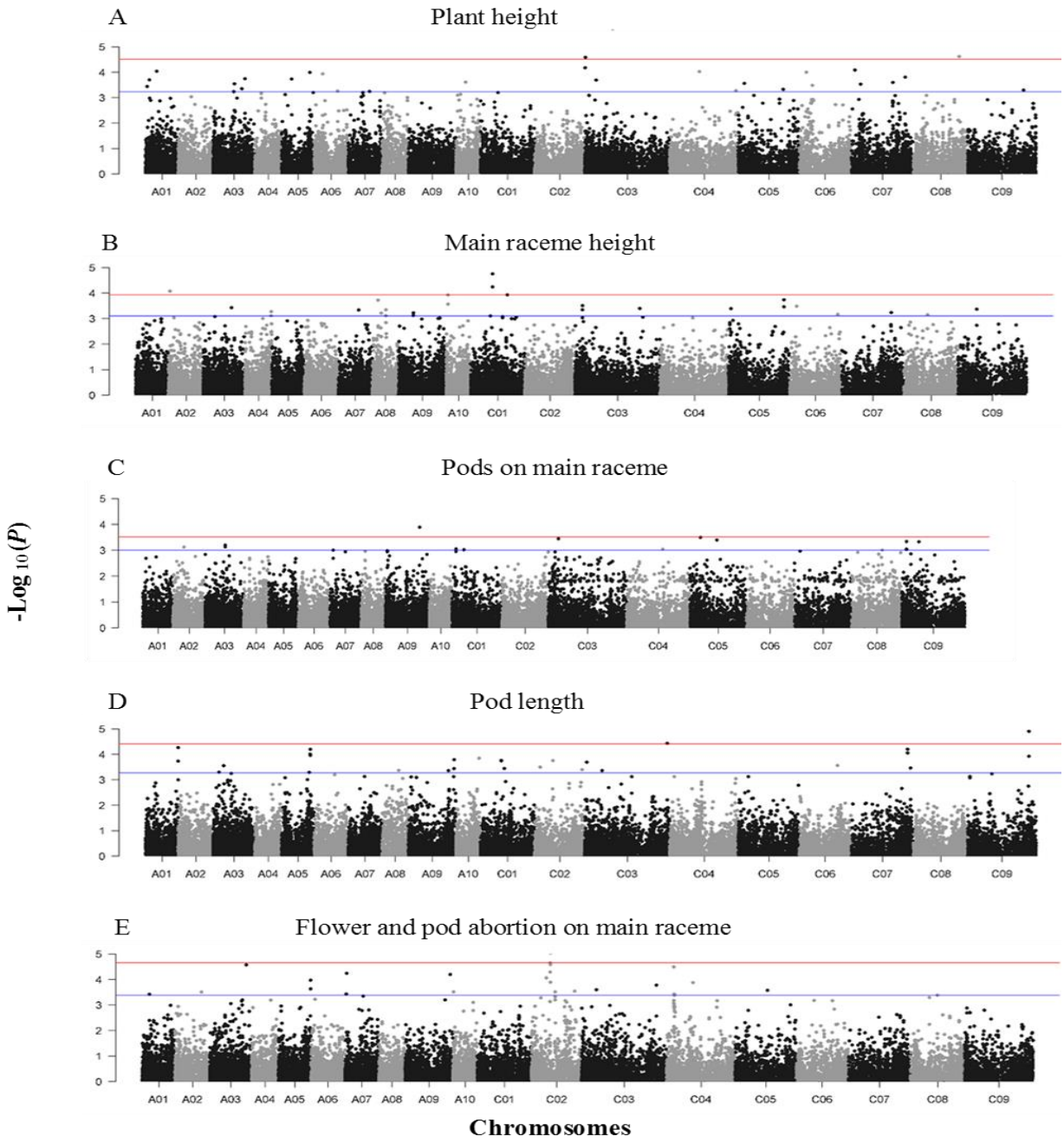


Figure 4.6. Manhattan plots showing *P*-values of markers across 19 chromosomes associated with five different traits (A-E). (A) Plant height, (B) main raceme height, (C) number of pods on main raceme, (D) pod length, and (E) pod abortion on main raceme. The *P* values are plotted on $\log_{10}$ scale and the markers are considered significant at $P \leq 0.001$.

Table 4.3. Significant markers associated with heat stress tolerance of five traits under field condition.

Marker					Allele 1			Allele 2			Heterozygous Allele		
	Chr	Pos	P	R^2 (%)	Allele	# Obs	Mean	Allele	# Obs	Mean	Allele	# Obs	Mean
Plant height (cm)													
chrC08_32368215	C08	32368215	2.40E-05	24	A	1	134.0	G	77	96.8	R	7	92.4
chrC03_545192	C03	545192	2.61E-05	24	G	52	94.1	T	18	103.58	K	15	98.5
chrCnn_rand_78509836	Cnn-rand	78509836	2.99E-05	23	C	7	98.4	T	72	95.1	Y	6	116.5
Raceme height (cm)													
chrC01_15689071	C01	15689071	1.74E-05	22	G	59	39.7	T	20	36.86	K	6	51.7
chrC01_15689086	C01	15689086	5.77E-05	20	C	55	40.0	T	25	37.5	Y	5	50.1
chrA02_1133295	A02	1133295	8.39E-05	20	A	25	37.5	T	55	40.0	W	5	50.1
chrC01_26101660	C01	26101660	1.18E-04	19	A	25	37.5	T	55	40.0	W	5	50.1
#Pods on main raceme													
chrA10_rand_2092893	A10_rand	2092893	9.42E-05	21	A	43	27.9	G	28	30.0	R	14	37.0
chrA10_rand_2092900	A10_rand	2092900	9.42E-05	21	C	62	30.7	T	12	33.1	Y	11	23.0
chrA09_26370461	A09	26370461	1.27E-04	21	A	71	29.0	T	2	27.0	W	12	37.1
chrAnn_rand_10002128	Ann-rand	10002128	2.98E-04	19	C	2	27.0	G	71	29.0	S	12	37.1
chrAnn_rand_10002131	Ann-rand	10002131	2.98E-04	19	A	11	32.5	G	64	30.8	R	10	22.6
Pod length (cm)													
chrC02_33478452	C02	33478452	7.34E-06	26	A	26	6.4	G	47	6.9	R	12	5.87
chrC09_43471822	C09	43471822	1.24E-05	25	A	25	6.5	G	44	6.9	R	16	5.97
chrAnn_rand_11544915	Ann-rand	11544915	3.50E-05	23	A	38	6.6	G	39	6.8	R	8	5.62
chrC03_58651519	C03	58651519	3.72E-05	23	A	11	7.0	G	68	6.7	R	6	5.37
Pod abortion													
chrA03_4072206	A03	4072206	5.20E-06	27	A	9	14.9	T	40	10.1	W	36	8.06
chrC02_13281695	C02	13281695	9.16E-06	26	A	16	7.7	G	20	13.5	R	49	8.90
chrC02_13209276	C02	13209276	2.22E-05	23	A	70	8.9	C	4	9.0	m	11	15.6
chrC02_13209244	C02	13209244	2.22E-05	23	C	4	9.0	T	70	8.9	Y	11	15.6
chrA10_1216770	A10	1216770	1.19E-04	16	C	67	39.9	G	3	29.8	S	15	41.9

Table 4.4. Significant Markers and QTL associated with total phenotypic variation of five different traits.

Trait	# of significant markers	# of QTL	Chromosomes	Position (Mbp)	% Phenotypic Variation
Plant height	38	6	A01	2.76	52.19
			C03	0.54	
			C06	5.17	
			C07	38.5	
			C07	6.80	
			C08	32.3	
Main raceme height	36	11	A02	1.13	71.75
			A03	19.9	
			A10	1.21	
			C01	15.6	
			C01	26.1	
			C05	39.3	
			C05	1.57	
			C07	35.3	
			C08	16.8	
			Cnn_ rand	67.4	
			Cnn_ rand	22.2	
Pods per main raceme	25	7	A09	26.3	53.21
			A10_ rand	2.09	
			Ann_ rand	10.0	
			C01	3.05	
			C01	9.23	
			C03	8.00	
			C09	3.59	
Pod length	38	11	A03	4.12	73.48
			A05	20.3	
			A09	32.4	
			A10	16.4	
			C01	14.8	
			C01	16.9	
			C02_ rand	3.64	
			C03	1.38	
			C03	12.3	
			C07	40.1	
			C09	43.4	
Pod abortion	35	7	A05	22.8	61.02
			A07	1.11	
			C02	13.2	
			C04	5.45	
			C04	5.46	
			C04_rand	0.98	
			C05	22.9	

Four markers significant at 0.001 percentile ($p \leq 8.39E-05$ (Table 4.3; Fig 4.6B) were identified for main raceme height. These significant markers were located on chromosome A02 (1.13 Mbp) and C01 (15.6 and 26.1Mbp). Thirty-two other markers were found significant at 0.01 percentile tail of the empirical distribution ($p \leq 7.84E-04$) (Table A13). Eleven QTL regions were identified through stepwise regression. These 11 QTL explained 71.75 % phenotypic variation and found on chromosome A02, A03, A10, C01, C05, C07, and C08 (Table 4.4). Many candidate genes such as plant calmodulin-binding protein is associated with Ca2+ binding and plant growth and development, indole acetic acid-induced protein 10 that enhance plant growth under drought stress condition, protein kinase family protein are involved in stem elongation and vascular development, ACC oxidase1 that favors plant growth and lowering stress susceptibility are associated with the QTL responsible for raceme height (Table A14).

In a number of pods on main raceme, five markers were found significant at the 0.001 percentile ($p \leq 2.98E-04$; Table 4.3; Fig. 4.6C). These markers were distributed on chromosome A09 (26.3 Mbp) and randomly distributed on chromosome A10 (20.9 Mbp). The other two markers were distributed anonymously. Besides these markers, 20 more markers were found significant at 0.01 percentile tail of the empirical distribution ($p \leq 9.86E-04$) (Table A13). Further, 7 major QTL were identified through stepwise regression which were responsible for 53.21% phenotypic variation of pods on main raceme (Table 4.4). Among them, 5 QTL were located on chromosomes A09, C01, C03 and C09, and A10_rand. Multiple candidate genes such as adenine nucleotide alpha hydrolases-like superfamily protein known to be involved in male sterility, protein kinase superfamily protein involved in pollen abortion, pyruvate kinase family protein, associated with early embryo abortion, proline-rich family protein associated with flower and pod development is present in the QTL regions (Table A14).

Pod length was found associated with four markers at 0.001 percentile tail of significance level ($p \leq 3.72E-05$; Table 4.3; Fig 4.6D), and these markers were located on chromosome C02 (33.4 Mbp), C03 (58.6 Mbp) and C09 (43.4 Mbp). One significant marker (Ann_rand, 11.5Mbp) was also found randomly and anonymously distributed within the genome. The other 34 markers were found significant at 0.01 percentile tail of the empirical distribution ($p \leq 9.87E-05$) (Table A13). Stepwise regression reveals that 73.48% variation was caused by the 11 major QTL (Table 4.4). These QTL were located on A03, A05, A09, A10, C01, C03, C07, C09 chromosomes and randomly distributed on C02 chromosomes. Multiple genes such as cellulose synthesis like A14 known to be involved in the young seedpod development, plant self-incompatibility protein S1 family that severely reduced pollen coats and cause male sterility, glutamine synthetase 1:4 which is involved in B-deficiency and pod development are present in the QTL region (Table A14).

Pod abortion was found associated with four significant markers and these markers were found significant at the 0.001 percentile ($p \leq 5.20E-06$; Table 4.3; Fig. 4.6E). The markers were located on chromosome A03 (4.07 Mbp) and C02 (13.20 to13.28 Mbp). Further 31 markers were identified with significant at 0.01 percentile tail of the empirical distribution ($p \leq 2.57E-05$) (Table A13). These markers were distributed on multiple chromosomes. A stepwise regression was performed, and 7 QTL regions were identified explaining 61.02% phenotypic variation of pod abortion (Table 4.4). Many candidate genes known to be involved in organ abortion were identified (Table A14). These genes included heat shock proteins, genes associated with male sterility, embryo abortion, pollen abortion, and reduced flowering fertility.

4.5. Discussions

Canola is the second largest oil producing crops in the world with low percentage of erucic acid and high percentage of unsaturated fatty acid, which is good for human health to reduce the risk of cardiovascular disease (Connor, 2000). It is a cool season crop and sensitive to heat stress (Morrison, 1993). Since heat stress is a growing concern, improvement of traits like heat stress tolerance in Canola might be an important criterion to expand the cultivation of this crop in the United States. As a part of this view, a genome-wide association study was conducted to identify significant markers closely associated with heat stress related traits that can be helpful for marker assisted selection program. The germplasms flowered within 40-60 days of planting and were considered as spring type. These plants are subject to heat stress during its reproductive developmental stage.

There are several studies on heat stress under controlled conditions, but limited studies on plant height, raceme height, total number of pods on main raceme, pod length as well as pod abortion of canola in natural condition in the field with heat stress. The germplasm used here has higher genetic diversity and a better mapping resolution that can be helpful to narrow QTL regions that can be used for marker assisted selection (MAS).

We studied plant height, main raceme height, number of pods per raceme, pod length as well as pod abortion of canola under natural heat stress conditions in the field. This study was conducted in summer 2014. Many researchers have conducted genome-wide association mapping study based on single year data. Hwang et al. (2014) conducted a genome-wide association study of seed protein and oil content in soybean with one year field trial in 2003. Zegeye et al. (2014) conducted association mapping on seedling and adult plant resistance to stripe rust in synthetic hexaploid wheat using single year data. Bellucci et al. (2015) conducted a single year field trial

in 2008 for association mapping in Scandinavian winter wheat for seed yield, plant height, and traits important for second-generation bioethanol production.

We had recorded data of plant height and raceme height which were varied significantly from different genotypes. Heat stress affects plant height and inflorescence height through reducing photosynthesis, which is one of the most heat sensitive physiological processes in plants (Yamamoto et al., 2008).

Heat stress causes pod sterility and pod abortion (Morrison, 1993). Variable flower and pod abortion were observed in different germplasms in our study. Variability of pod abortion due to heat stress is also reported in different crops such as tomato (Levy, Rabinowitch and Kedar 1978; Abdul-Baki, 1991), *Capsicum annum* L. (Rylski, 1986; Erickson & Markhart, 2002), bean (Konsens, Ofir & Kigel, 1991), cowpea (Craufurd et al., 1998), pea (Wery and Tardieu, 1997), and cotton (Reddy et al., 1992). Heat stress affects the tapetum layer of pollen and causes the significant changes in the tapetum layer which affects the nutrition supply, especially callus supply of pollen during microspore development. These shortage nutrient supply strongly affect the male gametogenesis therefore, the correct formation of microspore cells development is hampered which ultimately results in sterile mutants and pod abortion (Ma et al., 2005).

Heat stress tolerance is governed by multiple gene and many biochemical and metabolic pathways are involved with this abiotic stress. Different antioxidant activity, membrane lipid unsaturation, gene expression and protein translation, stability of protein, and accumulation of compatible solutes are also playing a significant role in heat stress tolerance (Kaya et al., 2001). Heat stress has a significant role in growth, development and reproduction of *Brassica* (Morrison, 1993; Angadi et al., 1999; Nuttal, 1992). Although this crop suffers from different

biotic and abiotic stress, however, heat stress cause significant damage on different phenotypic changes through genetic alteration of the crop.

In this study, genome-wide association mapping was conducted to identify significant markers associated with five traits related to heat-stress. GWAS helps to identify the candidate gene regions for each trait of interest depending on the marker number, trait, size and resolution of the population. It is also a powerful tool to identify QTL and candidate genes associated with specific traits of crop species (Huang et al., 2012). The phenotypic variation of many complex traits is influenced by the QTL where association mapping helps to find out molecular markers which are closely linked to the traits of interest or closely associated with the QTL or genes controlling the traits (Li et al., 2011). We have identified single nucleotide polymorphism (SNP) markers for different traits. SNPs are the frequently used marker which are contributing the majority of genotyping work in different crop species including *B. napus* (Trick et al., 2009). GWAS of canola using SNP and wide accessions of germplasms as mapping populations can therefore be helpful to find out significant markers and the candidate genes associated with the traits (Hasan et al., 2008; Li et al., 2014). It could also be helpful to identify a significant marker for the marker assisted selection breeding in canola.

About 37K cleaned SNP markers were used in this study. The missing data of the SNPs were further imputed following FastPHASE 1.3 (Scheet et al., 2006) and minor allele frequency (<0.05) to increase the map resolution of the study and to map the causal variant of the analysis. To protect from spurious marker-trait associations (Pressor et al., 2006; Price et al., 2010), we tested six regression models (Naïve, PC_3, PC_{17}, kinship, PC_3+kinship, and PC_{17}+ Kinship) that include structure and/or relatedness. The best model was selected on the basis of *P*-value of mean square difference (Mamidi et al., 2011). Among the six models tested in the study, the

model containing the lowest *P*-value was the best model for the respective trait to control the population structure.

In association mapping there is a chance of false positive association. Multiple testing corrections (Benjamini et al., 2001) were used to eliminate false positive associations. A Manhattan plot was constructed for each trait and initially on the basis of *P*-value that was estimated on the basis of 0.001 cutoff, bootstrapping and finally with the multiple testing corrections. Initially large number of significant markers were found associated with the heat stress traits when the p-value was P=0.001, but after multiple testing correctness and bootstrapping (Mamidi et al., 2014) only a few markers were found significantly associated with the QTL related to heat stress traits. Stepwise regression was performed to identify the minimum number of markers (Mamidi et al., 2014) associated with each trait that controlled by the major QTL which could be used for marker assisted selection.

Plant height is an important trait of canola affected by heat stress. Heat stress affects the photosynthesis (Crafts-Brandner et al., 2002) and produce Reactive Oxygen Species (ROS) Hasanuzzaman et al., 2013) which severely affect plant growth and development. Plants accumulate protein and osmolytes under heat stress, which helps to continue the photosynthesis through enhancing the activities of many antioxidants like superoxide dismutase (SOD), catalase (CAT) and peroxidise (POD) as well as scavenging the harmful ROS (Warich et al., 2012). QTL plays a significant role in phenotypic variation of particular traits of plants (Wang et al., 2013, Lorkovic, 2009, Tapia-López et al., 2008, Wu et al., 2001). Several studies identified QTL associated with heat stress in various crops, such as rice (Ye et al., 2012), cowpea (*Vigna unguiculata*) (Lucas et al., 2013), and tomato (Grilli et al., 2007) with a phenotypic variation between 2 and 20%, which is related to this study of QTL identification in *Brassica.* In our

study, the phenotypic variation of plant height due to the major QTL was about 53% indicates that the QTL plays a major role of plant development in *B. napus*. The *Brassica* gene model was used to identify the candidate genes associated with marker and heat stress. The significant marker was selected around 100 kB of each side of the major QTL due to the lower LD of the studied canola accessions (Michalak et al., Unpublished) and the position of the markers and the QTL as well as candidate genes are described based on the canola genome sequence published (Chalhoub et al., 2014). Many genes related to plant height under heat stress have been identified. The heavy metal transport/detoxification superfamily protein gene was found in chromosome C03 which was only 4 kb apart from the major QTL chrC03_545192. This gene is associated with plant growth and development and helps the plant to sustain under abiotic stress conditions (Hall 2002). Many other genes were found associated with heat stress such as H(+)-ATPase 2 which is involved in plant growth and development (Schubert, 2013); gibberellin 2-oxidase 8 which regulates plant growth (Fang Lo et al., 2008); ethylene-regulated nuclear protein (ERT2) which regulates plant growth and development through cell elongation, cell division, etc. (Sakai et al., 1998); and ABC-2 type transporter family protein, which is involved in plant growth, development and response to abiotic stresses (Kang et al., 2011). Other genes associated with plant growth and development like C2H2-like zinc finger protein (Chrispeels et al., 2000), iron regulated 2 (Yang et al., 2013);and Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein (Lin et al., 2015), were found in this study too.

Raceme height is correlated with the plant height that is ultimately associated with yield of canola. GWAS revealed 36 significant SNP markers located on eleven QTL on chromosome A02, A03, A10, C01, C05, C07 and C08 associated with raceme height. Many candidate genes were identified associated with raceme height involved in different physiological process. Of

these candidate genes, Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family proteins are involved in plant development (Lin et al., 2015), Plant calmodulin-binding protein is associated with Ca2+ binding and plant growth (Ranty et al., 2006), indoleacetic acid-induced protein 10 which enhances plant growth under drought stress condition (Yasin Ashraf et al., 2006), protein kinase family protein is involved in stem elongation and vascular development (Matschi et al., 2013), auxin response factor 1 that regulates plant growth and development (Li et al., 2016), mitogen-activated protein kinase that act as signal transporter for cell division and plant growth (Sinha et al., 2011), AP2/B3-like transcriptional factor family protein is involved in plant growth (Song et al., 2013), ACC oxidase 1 is involved in plant growth and lowering stress susceptibility (Van de poel, 2014).

Number of pods per plant depends on the pod development and the rate of abortion of pods per plant. Pollination and fertilization is the prerequisite for the pod development of crops. Heat stress affects the pollination of *Brassica* through the desiccation of pollen and reduction the pollen receptivity of the stigma (Rao et al., 1992). Many genes are involved in the variation of number of pods per plant. We had identified seven significant QTL those explained 53.21% phenotypic variation of number of pods per main raceme. Twenty-five significant markers were identified associated with these QTL which are located on the chromosome A09, C01, C03 and C09. The closest marker chrC03_8003052 is located on *Brassica* gene BnaC03g15870D that contain protein kinase superfamily protein, which is involved in pollen abortion of crops (Radchuk et al., 2006). Three significant marker chrA10_rand_2092900, chrC01_3055220 and chrA09_26370461 were found on the chromosome A10, C01 and A09, respectively. These markers are located on 4, 6, and 7 kb apart from the target *Brassica* genes, respectively. Many candidate genes were identified associated with the variation of number of pods per plants.

Among the candidate genes, basic helix-loop-helix (bHLH) DNA-binding superfamily protein is involved in the development and dehiscence of seed and pod (Hudson et al., 2015), Protein kinase superfamily protein is involved in pollen abortion (Radchuk et al., 2006), Pyruvate kinase family protein is associated with early embryo abortion of flower (Zhang et al.,2014), ARM repeat superfamily protein is involved in self-incompatibility and reduction of pod number (Sharma et al., 2016), Chaperone DNAJ-domain superfamily protein, which is involved in male sterility to reduce pod number (Yang et al., 2009), DNAJ heat shock N-terminal domain-containing protein that makes tolerant to heat and prevent fruit drop (Zhao-Xia Ma et al., 2015), proline-rich family protein is associated with flower and pod development (Girno et al., 2013), adenine nucleotide alpha hydrolases-like superfamily protein is involved in male sterility (Mok et al., 2001), Homeodomain-like protein, regulate anther dehiscence (Wilson et al., 2011), Cytochrome P450 is involved in the pollen tube development and fertilization (Zhao et al., 2015), Pyruvate kinase family protein is found associated with early embryo abortion (Zhang et al., 2014).

Pod length is one of the indicators of seed yield of *Brassica*. The pod length is also affected by heat stress. High temperature reduces the photosynthetic capacity (Crafts-Brandner et al., 2002) and also causes pollen abortion (Zhang et al., 2014) which affects the growth and development of the pod. Thirty-eight significant markers located on 11 QTL associated with pod length in relation to heat stress were identified in this study. The QTL are located on the chromosome A03, A05, A09, A10, C03, C07 and C09 with a phenotypic variation of 73.48%. The marker chrA03_4124353 located on chromosome A3 is only 1 kb away from *Brassica* gene BnaA03g09160D (Cysteine/Histidine-rich C1 domain family protein). This gene is involved in tapetal development, Programmed Cell Death (PCD) and pollen grain sterility (Zhang et al.,

2014). Many of other genes such as 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein (Leisner et al., 2014), Cysteine/Histidine-rich C1 domain family protein (Zhang et al., 2014), Heat shock protein 18.2 (Kim et al., 2001), Zinc finger (C3HC4-type RING finger) family protein (Wu et al., 2014), Cellulose synthase like A14 (Park et al., 2013), Homeodomain-like superfamily protein (Wilson et al., 2011), Syntaxin of plants 71 (Sharma et al., 2014), Cellulose synthase 5 (Park et al., 2013), Plant self-incompatibility protein S1 family (Samuel et al., 2009), Cytochrome P450 (Zhao et al., 2015), Ubiquitin family protein (Mazzucotelli et al., 2006), Malectin/receptor-like protein kinase family protein (Matschi et al., 2013), Glutamine synthetase 1;4 (Bargaz et al., 2015), Auxin response factor 19 (Li et al 2016), AGAMOUS-like 24 (Yu et al 2002), P450 reductase 1 (Bak et al., 2011) were also identified associated with the cytoplasmic male sterility, pollen tube and pollen coat development, Boron deficiency, and seed pod development

Pod abortion is an important phenotypic trait of heat stress, which causes significant yield loss of *Brassica*. Thirty-five SNPs were identified associated with pod abortion on different chromosomes. Stepwise regression identified seven significant QTL located on chromosome A05, A07, C02, C04 and C05. Stepwise regression is used to minimize the number of markers for QTL determination, estimating allelic combinations (Mamidi et al., 2014; Gurung et al., 2014). We had also detected the distance among the major QTL and the candidate genes associated with the traits. The closest markers chrC04_5456736, and ChrC04_rand_988002 were found 4 kb apart from *Brassica* gene BnaC04g07360D and BnaC04g01250D, respectively. Two other markers chrA05_22801086 and chrA05_22801086 were also found 5 and 6 kb apart from the *Brassica* gene BnaA05g33770D and BnaA05g33780D, respectively, which were located on the chromosome A05. The gene associated with these QTLs are F-box family protein, associated

with the reduction of flower fertility and reduced number of pod set (Ariizumi et al., 2011), cyclic nucleotide gated protein that is involved in meiotic division and fruit development (Yang et al., 2006), myb domain protein 57 associated with drought stress tolerance to reduce pod abortion (Baldoni et al., 2015) and, Adenine nucleotide alpha hydrolases-like superfamily protein that are involved in male sterility and ultimately cause pod abortion (Mok et al., 2001).

4.6. Conclusion

Eighty-five spring type *Brassica napus* accessions were evaluated for heat stress tolerant traits in the field. Under heat stress in the field the heat tolerant traits such as plant height was varied from 68-134 cm, main raceme height varied 15.5-61.1 cm, number of pods on main raceme varied 13.0-52.6, pod length varied 4.27- 8.21 cm, and pod abortion per main raceme varied from 1.68 - 30.1. Genome-wide association study was conducted using about 37K high quality cleaned single nucleotide polymorphic markers. 172, and 21 significant markers were identified associated with five different traits using $P = 0.1$ and 0.01 cutoff of 10,000 bootstraps, respectively. A total of 6, 11, 7, 11 and 7 QTL were identified which explained 52.19%, 71.75%, 53.21%, 73.48% and 61.02% phenotypic variation for plant height, main raceme height, pods on main raceme, pod length, and pod abortion per main raceme, respectively. Many candidate genes associated with the QTL were found to cause variation in plant height, main raceme height, number of pods per main raceme, pod length and, pod abortion of *Brassica*. The markers linked to the respective traits could help breeders to select high yielding heat stress tolerant canola variety through marker-assisted selection. As it was a one year study, so, marker might be helpful to recommend the selected markers for fruitful use in marker assisted selection.

4.7. References

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CHAPTER 5. SUMMARY

The study of heat stress tolerant traits of rapeseed/canola was conducted in controlled conditions in a greenhouse and growth chamber, and in the field with a wide collection of spring type germplasms. In the greenhouse, two sets of 88 accessions with three replications per set were grown at 22/18°C day/night temperatures. The germplasm in the set-1 was grown as a control experiment, whereas the germplasm in the set-2 at 6-day flowering stage were exposed to an artificial heat simulating condition for 5 days in a plant growth chamber. In the field, 85 accessions were allowed to expose under natural heat stress conditions. Genome wide association study was conducted using 37,539 high quality cleaned single nucleotide polymorphic (SNP) markers. Under heat simulating conditions, a total of 115 significant (-log10 *P*-value <0.01) markers associated with three traits were identified, whereas, 15 markers were found highly significant using *P*=0.001 thresh hold. After stepwise regression, a total of 5, 8, and 7 QTL were identified associated with pollen sterility, sterile/aborted pods, and number of pods on main raceme which explained 46.31%, 60.45% and 60.59% phenotypic variations, respectively. In the field study, 172 significant (-log10 *P*-value <0.01) markers were identified associated with five traits and, finally 21 markers were identified using *P*=0.001 cutoff. Stepwise regression identified 6, 11, 7, 11 and 7 QTL causes phenotypic variation of 52.19%, 71.75%, 53.21%, 73.48% and 61.02% for plant height, main raceme height, pods on main raceme, pod length, and pod abortion on main raceme, respectively. Two traits such as sterile/aborted pods on main raceme, and total number of pods on main raceme were common for both greenhouse and field studies. Three markers (chrC05_37931234, chrC05_42411802 and chrC05_22964001) linked to sterile/aborted pods on main raceme identified 3 QTL were located on chromosome C05. On the other hand, five markers (chrA10_1645036, chrA10_14378371, chrC03_23275707,

chrA10_rand_2092893, chrC03_8003052) identified 5 QTL associated with a total number of pods on main raceme located on Chromosome A10 and C03 was identified. Many candidate genes such as, Kinase superfamily protein, Cleavage and polyadenylation specificity factor (CPSF), A subunit protein, Calmodulin 7, ARM repeat superfamily protein, F-box family protein, Plant self-incompatibility protein S1 family, MATE efflux family protein, Pectin methyl esterase 31, Lipase/Acylhydrolase superfamily protein, C2H2-type zinc finger family protein, ABC-2 type transporter family protein, Indole acetic acid-induced protein 10, Pyruvate kinase family protein, heat shock protein 18.2, ARM repeat superfamily protein were identified associated with these QTL. The identified genes were found associated with male sterility, pollen abortion, embryo abortion as well as reduction of plant growth and development. The discovered major QTL could help breeders to select high yielding heat stress tolerant canola accessions through marker-assisted selection as well as to develop heat stress tolerant canola varieties for breeding program.

APPENDIX

Table A1. Heat Susceptibility Index (HSI) of the three different traits under controlled condition.

Traits	Av*	Stdev*	Skewness	Kurtosis	Kolmogorov-Smirnov *P* value
Pollen sterility	133.8	208.6	2.883	9.640	0.260
Sterile/aborted pods	298.7	287.5	0.621	-0.509	0.201
Pods on main raceme	1.375	1.367	0.729	-0.390	0.172

* Av=Average; Stdev*= Standard deviation

Table A2. ANOVA for the three different traits of *Brassica napus* under controlled condition.

Traits	SV	Df	SS	MS	F-value	Pr> F
Pollen sterility	Rep	2	1.9596068	0.979803	3.37	0.0368
	Gen	87	52.273896	0.600849	2.06	<.0001
Sterile pod	Rep	2	11.479924	5.739962	5.15	0.0067
	Gen	87	246.29832	2.831015	2.54	<.0001
Total pods on main raceme	Rep	2	175.15909	87.57955	0.91	0.4035
	Gen	87	35964.424	413.3841	4.31	<.0001

Table A3. ANOVA for the three different traits of *Brassica napus* under heat stress.

Traits	SV	Df	SS	MS	F-value	Pr> F
Pollen sterility	Rep	2	103.7782	51.8891	3.16	0.0450
	Gen	87	115983.9	1333.14	81.1	<.0001
Sterile pod	Rep	2	128.8484	64.4242	1.96	0.1439
	Gen	87	12485.12	143.507	4.37	<.0001
Total pods on main raceme	Rep	2	85.78030	42.8901	0.95	0.3885
	Gen	87	27622.14	317.495	7.04	<.0001

Table A4. ANOVA for the three different traits of *Brassica napus* across all environments.

Traits	SV	Df	SS	MS	F-value	Pr> F
Pollen sterility	Gen	87	58520.174	672.6456	80.400	<.0001
	Rep(Env)	4	105.73785	26.43446	3.1600	0.0143
	Env	1	56954.393	56954.393	6807.4	<.0001
	Env*Gen	87	57516.053	661.10407	79.020	<.0001
Sterile pods	Gen	87	58520.174	672.64568	80.400	<.0001
	Rep(Env)	4	140.32841	35.082100	2.0700	0.0849
	Env	1	21455.674	21455.674	1263.1	<.0001
	Env*Gen	87	5569.1241	64.012920	3.7700	<.0001
Total pods on main raceme	Gen	87	48471.573	557.14453	7.9000	<.0001
	Rep(Env)	4	260.93939	65.234850	0.9200	0.4497
	Env	1	3088.5018	3088.5018	43.780	<.0001
	Env*Gen	87	15114.998	173.73561	2.4600	<.0001

Table A5. Correlation among the traits under control and heat stress condition.

Traits	Control (GH)		Heat Stressed	
	Total pod	Sterile pod	Total pod	Sterile pod
Total pod	---------		--------	
Sterile pod	0.22*		0.28**	
Pollen sterility	0.084 ns	0.12 ns	0.026 ns	0.015 ns

*, and ** = significantly different at 0.05, and 0.01 levels of probability, respectively; ns=not significant at P=0.05.

Table A6. Significant markers associated with different traits under heat stress condition.

Pollen Sterility									
				Allele 1		**Het Allele**		**Allele 2**	
Marker	**Chr**	**Pos**	***P*-value**	**Obs**	**Mean**	**Obs**	**Mean**	**Obs**	**Mean**
chrA02_1481324	A02	1,481,324	7.33E-07	16	036	14	458	58	076
chrA02_16363471	A02	16,363,471	1.34E-07	75	105	03	542	10	052
chrA03_4168209	A03	4,168,209	4,168,209	12	202	13	181	63	086
chrA03_11267300	A03	11,267,300	11,267,300	78	095	01	1084	09	095
chrA03_11267316	A03	11,267,316	11,267,316	11	082	01	1084	76	098
chrA03_11485277	A03	11,485,277	11,485,277	83	100	01	1084	04	006
chrA03_11485282	A03	11,485,282	11,485,282	04	006	01	1084	83	100
chrA03_16097707	A03	16,097,707	16,097,707	82	103	01	1084	05	005
chrA03_26857689	A03	26,857,689	26,857,689	04	150	01	1084	83	091
chrA05_5780703	A05	5,780,703	5.26E-06	08	026	80	135	00	000
chrA06_10285855	A06	10,285,855	2.82E-07	21	128	01	1084	66	088
chrA06_10285865	A06	10,285,865	2.82E-07	66	088	01	1084	21	128
chrA06_20547923	A06	20,547,923	1.80E-06	03	000	04	592	81	101
chrA06_rand_1806137	A06_rand	1,806,137	1.86E-06	78	090	02	766	08	043
chrA07_rand_1794834	A07_rand	1,794,834	2.98E-07	23	165	64	080	01	1084
chrA08_10051973	A08	10,051,973	1.82E-06	04	011	01	000	83	124
chrAnn_rand_4645588	Ann_rand	4,645,588	1.35E-09	22	086	64	100	02	1084
chrC01_3445668	C01	3,445,668	1.90E-06	76	127	09	051	03	000
chrC01_6790079	C01	6,790,079	1.27E-07	66	097	21	090	01	1084
chrC01_21285298	C01	21,285,298	6.70E-07	02	1084	39	126	47	075
chrC01_33027305	C01	33,027,305	1.28E-07	40	100	47	090	01	1084
chrC01_35999141	C01	35,999,141	5.29E-06	78	078	05	452	05	241
chrC02_rand_1420921	C02_rand	1,420,921	2.77E-06	74	144	11	032	03	000
chrC02_rand_4413987	C02_rand	4,413,987	2.95E-07	01	1084	07	078	80	097

Table A6. Significant markers associated with different traits under heat stress condition (continued).

Pollen Sterility									
				Allele 1		**Het Allele**		**Allele 2**	
Marker	**Chr**	**Pos**	***P*-value**	**Obs**	**Mean**	**Obs**	**Mean**	**Obs**	**Mean**
chrC03_394217	C03	394,217	4.64E-08	01	1084	37	059	50	117
chrC03_27509176	C03	27,509,176	2.99E-07	01	1084	38	113	49	080
chrC03_31613088	C03	31,613,088	2.99E-07	81	095	01	1084	06	104
chrC04_47184437	C04	47,184,437	6.38E-08	01	1084	41	114	46	085
chrC04_47184438	C04	47,184,438	6.38E-08	01	1084	41	114	46	085
chrC04_rand_2348374	C04_rand	2,348,374	1.84E-06	54	092	28	088	06	383
chrC05_9566302	C05	9,566,302	1.73E-07	10	003	77	106	01	1084
chrC07_13100599	C07	13,100,599	1.91E-07	01	1084	36	072	51	111
chrC08_3759455	C08	3,759,455	1.42E-08	27	039	60	122	01	1084
chrCnn_rand_17510479	Cnn_rand	17,510,479	5.86E-06	02	605	61	087	25	108
chrCnn_rand_18549100	Cnn_rand	18,549,100	7.03E-08	06	542	53	099	29	097
chrCnn_rand_18549112	Cnn_rand	18,549,112	7.78E-11	04	1084	51	104	33	087
chrCnn_rand_18549122	Cnn_rand	18,549,122	7.03E-08	29	097	53	099	06	542
chrUnn_rand_6658728	Unn_rand	6,658,728	9.85E-07	15	056	66	100	07	389
chrUnn_rand_6711432	Unn_rand	6,711,432	4.29E-06	12	283	47	058	29	137
Sterile/ aborted Pods									
				Allele 1		**Het Allele**		**Allele 2**	
Marker	**Chr**	**Pos**	***P*-value**	**Obs**	**Mean**	**Obs**	**Mean**	**Obs**	**Mean**
chrA01_8090850	A01	8,090,850	4.16E-05	57	327	15	202	16	234
chrA01_21809368	A01	21,809,368	7.03E-04	38	242	39	230	11	468
chrA02_6194788	A02	6,194,788	8.01E-04	69	243	11	326	08	450
chrA02_22559756	A02	22,559,756	0.000354	73	258	05	435	10	349
chrA02_22559765	A02	22,559,765	0.000354	73	258	05	435	10	349
chrA02_22697009	A02	22,697,009	0.000344	12	348	03	614	73	258

Table A6. Significant markers associated with different traits under heat stress condition (continued).

Sterile/ aborted Pods									
				Allele 1		**Het Allele**		**Allele 2**	
Marker	**Chr**	**Pos**	***P*-value**	**Obs**	**Mean**	**Obs**	**Mean**	**Obs**	**Mean**
chrA03_13865121	A03	13,865,121	2.82E-05	19	458	15	451	54	218
chrA03_16335953	A03	16,335,953	3.33E-04	04	556	05	000	79	263
chrA03_18700126	A03	18,700,126	9.90E-04	71	251	07	366	10	348
chrA05_rand_2356690	A05_rand	2,356,690	6.29E-04	50	231	18	437	20	269
chrA06_12786141	A06	12,786,141	9.24E-04	03	000	06	575	79	278
chrA07_505308	A07	505,308	9.36E-04	78	287	06	001	04	230
chrA08_214273	A08	214,273	5.62E-04	78	274	03	409	07	288
chrA08_216429	A08	216,429	5.48E-04	08	288	02	320	78	275
chrA08_9665215	A08	9,665,215	2.55E-04	17	352	04	352	67	253
chrA08_9665217	A08	9,665,217	2.46E-04	18	338	04	352	66	254
chrA08_9665233	A08	9,665,233	2.27E-05	17	380	03	235	68	254
chrA08_9689294	A08	9,689,294	3.47E-04	12	351	06	441	70	245
chrA09_25264986	A09	25,264,986	2.44E-04	72	250	07	230	09	443
chrA09_27884724	A09	27,884,724	1.79E-04	75	258	07	422	06	351
chrA09_30791683	A09	30,791,683	6.49E-05	65	252	07	531	16	320
chrA09_32074221	A09	32,074,221	9.27E-04	72	271	07	026	09	443
chrA10_12793248	A10	12,793,248	4.83E-04	08	243	04	409	76	278
chrC01_38231402	C01	38,231,402	1.94E-05	33	241	22	168	33	393
chrC01_38249968	C01	38,249,968	8.64E-04	32	404	54	216	02	116
chrC03_947250	C03	947,250	7.98E-04	22	201	32	498	34	171
chrC03_12712425	C03	12,712,425	6.21E-04	40	281	11	355	37	260
chrC03_50178610	C03	50,178,610	8.12E-04	58	281	08	288	22	270
chrC03_50178612	C03	50,178,612	8.12E-04	22	270	08	288	58	281
chrC03_50178621	C03	50,178,621	9.67E-04	57	283	07	288	24	267
chrC04_254453	C04	254,453	5.05E-04	58	299	24	164	06	661

Table A6. Significant markers associated with different traits under heat stress condition (continued).

Sterile/ aborted Pods									
				Allele 1		**Het Allele**		**Allele 2**	
Marker	**Chr**	**Pos**	***P*-value**	**Obs**	**Mean**	**Obs**	**Mean**	**Obs**	**Mean**
chrC04_918148	C04	918,148	9.68E-05	11	358	10	288	67	255
chrC04_44274216	C04	44,274,216	5.44E-04	06	492	05	441	77	246
chrC05_37931234	C05	37,931,234	4.71E-04	30	101	33	439	25	256
chrC05_42411802	C05	42,411,802	7.70E-04	03	384	17	479	68	219
chrC07_27295639	C07	27,295,639	6.45E-04	09	313	04	614	75	264
chrCnn_rand_17211548	Cnn_rand	17,211,548	4.38E-04	66	265	05	518	17	270
chrCnn_rand_73794981	Cnn_rand	73,794,981	2.46E-04	17	237	11	239	60	306
chrUnn_rand_6711432	Cnn_rand	6,711,432	5.93E-04	12	491	47	130	29	426
Pods on main raceme									
				Allele 1		**Het Allele**		**Allele 2**	
Marker	**Chr**	**Pos**	***P*-value**	**Obs**	**Mean**	**Obs**	**Mean**	**Obs**	**Mean**
chrA01_21164074	A01	21,164,074	0.00155425	42	1.50	32	2.60	14	2.0
chrA02_3316315	A02	3,316,315	5.47E-05	79	1.9	04	3.5	05	3.6
chrA02_3785127	A02	3,785,127	4.65E-04	03	4.6	04	3.5	81	1.9
chrA02_5382625	A02	5,382,625	0.001248573	82	1.9	03	3.5	03	3.7
chrA02_5589336	A02	5,589,336	2.09E-04	09	3.2	03	00	76	1.8
chrA03_25984973	A03	25,984,973	3.66E-04	02	0.0	14	2.8	72	1.9
chrA04_15181007	A04	15,181,007	1.95E-04	61	1.8	24	2.4	03	4.0
chrA04_15181010	A04	15,181,010	1.95E-04	03	4.0	24	2.4	61	1.8
chrA05_2492635	A05	2,492,635	7.74E-04	23	1.7	17	3.2	48	1.8
chrA05_10441722	A05	10,441,722	7.09E-04	04	0.9	07	3.7	77	1.9
chrA05_15015248	A05	15,015,248	0.001580437	02	2.7	37	1.7	49	2.3
chrA05_15015254	A05	15,015,254	0.001580437	49	2.3	37	1.7	02	2.7
chrA05_15015285	A05	15,015,285	0.001580437	49	2.3	37	1.7	02	2.7

Table A6. Significant markers associated with different traits under heat stress condition (continued).

Pods on main raceme									
				Allele 1		**Het Allele**		**Allele 2**	
Marker	**Chr**	**Pos**	***P*-value**	**Obs**	**Mean**	**Obs**	**Mean**	**Obs**	**Mean**
chrA01_21164074	A01	21,164,074	0.00155425	42	1.50	32	2.60	14	2.0
chrA06_19592145	A06	19,592,145	0.001387125	63	2.2	07	1.5	18	1.8
chrA06_20870206	A06	20,870,206	9.02E-05	08	3.2	06	00	74	1.9
chrA06_20874104	A06	20,874,104	0.001266024	05	2.7	08	2.8	75	1.9
chrA06_20955765	A06	20,955,765	1.37E-04	05	3.1	06	00	77	2.0
chrA06_22014672	A06	22,014,672	0.001500718	80	2.0	01	00	07	2.7
chrA07_23503722	A07	23,503,722	3.05E-04	05	2.6	07	3.3	76	1.8
chrA10_1645036	A10	1,645,036	1.39E-04	11	2.5	43	1.7	34	2.3
chrA10_12998203	A10	12,998,203	0.00117271	44	1.8	11	1.7	33	2.4
chrA10_14378371	A10	14,378,371	5.31E-04	73	2.1	09	3.1	06	1.1
chrA10_14851252	A10	14,851,252	5.14E-04	02	3.3	05	3.9	81	1.9
chrA10_14871336	A10	14,871,336	0.001511636	79	1.9	08	3.3	01	0.0
chrAnn_rand_4588189	Ann_rand	4,588,189	0.001575634	31	2.0	51	2.1	06	2.6
chrC02_rand_3127972	C02_rand	3,127,972	0.001304941	68	1.9	04	4.6	16	2.1
chrC02_rand_3127976	C02_rand	3,127,976	0.001304941	68	1.9	04	4.6	16	2.1
chrC03_23275707	C03	23,275,707	0.001561324	20	1.9	35	2.6	33	1.6
chrC07_34885819	C07	34,885,819	0.001516786	10	3.3	17	1.6	61	1.9
chrC09_41084846	C09	41,084,846	3.97E-04	01	0.0	09	3.3	78	1.9
chrCnn_rand_8679255	Cnn_rand	8,679,255	4.28E-04	08	1.6	58	2.1	22	2.0
chrCnn_rand_8679287	Cnn_rand	8,679,287	0.001306417	09	1.9	59	2.1	20	2.0
chrCnn_rand_12757556	Cnn_rand	12,757,556	0.001302798	04	2.7	02	4.6	82	1.9
chrCnn_rand_35427743	Cnn_rand	35,427,743	4.88E-05	80	1.9	03	3.5	05	3.6
chrCnn_rand_36648262	Cnn_rand	36,648,262	0.001729644	44	2.1	39	1.8	05	3.7
chrCnn_rand_55711143	Cnn_rand	55,711,143	0.001667809	15	3.2	12	1.2	61	2.0
chrCnn_rand_55825162	Cnn_rand	55,825,162	7.00E-04	79	2.1	05	0.6	04	3.4

Table A7. Candidate genes associated with the QTL related to different traits under heat stress condition.

Gene model	Chromosome	start	end	Gene annotation	Gene function	References
Pollen sterility						
GSBRNA2T00143205001	chrA02	1,381,512	1,384,147	glutamine synthetase 1;4	Oxidative stress, and B-defficiency	Bargaz et al. (2015)
GSBRNA2T00143187001	chrA02	1,451,304	1,454,179	Protein kinase superfamily protein	pollen abortion	Radchuk et al. (2006)
GSBRNA2T00143186001	chrA02	1,454,367	1,456,043	heat shock factor 3	Associated with male sterility	Kim et al. (2001)
GSBRNA2T00143181001	chrA02	1,469,412	1,475,092	cellulose-synthase like D2	Associated young seedpod development	Park et al. (2013)
GSBRNA2T00143169001	chrA02	1,526,097	1,528,165	Pectin lyase-like superfamily protein	Involved in pollen tube growth	Zhao et al. (2015)
GSBRNA2T00143164001	chrA02	1,540,681	1,542,666	Cyclin A1;1	Involved in meiotic division in rice	Yang et al. (2006)
GSBRNA2T00143160001	chrA02	1,551,186	1,553,704	Homeodomain-like superfamily protein	Regulate anther dehiscence	Wilson et al. (2011)
GSBRNA2T00143159001	chrA02	1,555,660	1,559,910	homeodomain GLABROUS 9	Regulate anther dehiscence	Wilson et al. (2011)
GSBRNA2T00143158001	chrA02	1,564,900	1,568,272	glutamate decarboxylase	Involved in pollen tube growth in arabiodiopsis	Palanivelu. (2003)
GSBRNA2T00143152001	chrA02	1,579,851	1,584,847	Cellulose synthase family protein	Associated young seedpod development	Park et al. (2013)
GSBRNA2T00125949001	chrC01	21,297,841	21,299,412	cytochrome P450, family 71, subfamily A, polypeptide 23	pollen abortion with reduced number of elongated siliques	Bak et al. (2011)
GSBRNA2T00125953001	chrC01	21,336,887	21,338,435	cytochrome P450, family 71, subfamily A, polypeptide 23	pollen abortion with reduced number of elongated siliques	Bak et al. (2011)
GSBRNA2T00098391001	chrC02_random	1,363,612	1,369,466	Calmodulin-binding transcription activator protein	Involved in embryo development	Radchuk et al. (2006)
GSBRNA2T00047754001	chrC05	9,663,726	9,665,733	acyl activating enzyme 1	Involvement Pollen development and fertilization	Souza et al. (2009)
GSBRNA2T00019534001	chrUnn_random	6,778,955	6,779,833	F-box/RNI-like/FBD-like domains-containing protein	Involved in floral organ development	Jain et al. (2007)

Table A7. Candidate genes associated with the QTL related to different traits under heat stress condition (continued).

Sterile/ aborted pods	Chromosome	start	end	Gene annotation	Gene function	References
GSBRNA2T00149519001	chrA01	8,156,639	8,158,938	Phosphatidate cytidylyltransferase family protein	Involved in Microspore development	Yamaoka et al. (2011)
GSBRNA2T00149515001	chrA01	8,169,399	8,171,514	Pentatricopeptide repeat (PPR) superfamily protein	Leads to embryo abortion	Lurin et al. (2013)
GSBRNA2T00137595001	chrA03	16,296,098	16,301,133	phosphoenolpyruvate carboxylase 3	Associated with seed abortion	Fischinger et al. (2010)
GSBRNA2T00137594001	chrA03	16,301,210	16,303,561	Galactosyltransferase family protein	Involved in ovule abortion and reduced seed set	Basu et al. (2015)
GSBRNA2T00137589001	chrA03	16,327,693	16,328,656	RAB GTPase homolog A1G	essential for male fertility	Gutkowska et al. (2014)
GSBRNA2T00137582001	chrA03	16,351,813	16,353,433	NAC (No Apical Meristem) domain transcriptional regulator superfamily protein	Associated with stress response	Jin et al. (2013)
GSBRNA2T00137580001	chrA03	16,358,258	16,359,218	ethylene responsive element binding factor 4	Associated with Number of Seeds per Pod	Kagale et al. (2010)
GSBRNA2T00137560001	chrA03	16,433,475	16,434,855	indole-3-acetic acid inducible 19	induce abortion of flowers	Nico et al. (2015)
GSBRNA2T00057373001	chrA09	31,985,235	31,990,347	aspartic proteinase A1	Involved in Pod and seed development	Chen et al. (2002)
GSBRNA2T00057374001	chrA09	31,990,456	31,992,915	F-box family protein	Reduced flower fertility	Ariizumi et al. (2011)
GSBRNA2T00057385001	chrA09	32,017,249	32,018,865	FAD-binding Berberine family protein	Drymatter accumulation and seed development	Zhao et al. (2015)
GSBRNA2T00057386001	chrA09	32,019,986	32,020,405	Plant self-incompatibility protein S1 family	severely reduced pollen coats and cause male sterility	Samuel et al. (2009)
GSBRNA2T00057388001	chrA09	32,022,888	32,023,304	Plant self-incompatibility protein S1 family	severely reduced pollen coats and cause male sterility	Samuel et al. (2009)
GSBRNA2T00057391001	chrA09	32,034,286	32,037,146	MATE efflux family protein	Involved in embryo abortion	Zhao et al. (2015)

Table A7. Candidate genes associated with the QTL related to different traits under heat stress condition (continued).

Sterile/ aborted pods	Chromosome	start	end	Gene annotation	Gene function	References
GSBRNA2T00057396001	chrA09	32,054,111	32,056,812	Protein kinase superfamily protein	pollen abortion in barley	Radchuk et al. (2006)
GSBRNA2T00057403001	chrA09	32,114,727	32,115,889	syntaxin of plants 124	Involved in pollen tube growth	Sharma et al. (2014)
GSBRNA2T00057408001	chrA09	32,131,104	32,132,634	F-box and associated interaction domains-containing protein	Involved in floral organ development	Jain et al. 2007
GSBRNA2T00057419001	chrA09	32,167,384	32,167,734	F-box and associated interaction domains-containing protein	Involved in floral organ development	Jain et al. (2007)
GSBRNA2T00065907001	chrA10	12,703,569	12,705,505	calcium exchanger 7	Involved in pod development and inhibit elongation of gynophores	Zhao et al. (2015)
GSBRNA2T00065896001	chrA10	12,766,271	12,769,875	MATE efflux family protein	Involved in embryo abortion	Zhao et al. (2015)
GSBRNA2T00065871001	chrA10	12,827,579	12,829,368	RGA-like protein 3	induced seed abortion	Cheng et al. (2015)
GSBRNA2T00065861001	chrA10	12,860,636	12,861,656	Adenine nucleotide alpha hydrolases-like superfamily protein	Involved in male sterility	Mok et al. (2001)
GSBRNA2T00135353001	chrA10	12,883,556	12,885,711	Homeodomain-like superfamily protein	Regulate anther dehiscence	Wilson et al. (2011)
GSBRNA2T00106056001	chrC05	37,845,107	37,847,738	Protein kinase superfamily protein	pollen abortion in barley	Radchuk et al. (2006)
GSBRNA2T00106048001	chrC05	37,867,378	37,867,917	Zinc finger, C3HC4 type (RING finger) family protein	cellular regulation in plants	Wu et al. (2014)
GSBRNA2T00106046001	chrC05	37,876,691	37,880,376	pectin methylesterase 3	Involved in pollen tube growth	Zhao et al. (2015)
GSBRNA2T00106034001	chrC05	37,935,924	37,937,363	GDSL-motif lipase 4	plays an important role in embryo abortion	Zhao et al. (2015)
GSBRNA2T00023867001	chrC05	38,023,921	38,024,922	Pentatricopeptide repeat (PPR) superfamily protein	Leads to embryo abortion	Lurin et al. (2013)
GSBRNA2T00023866001	chrC05	38,025,038	38,025,792	Pentatricopeptide repeat (PPR) superfamily protein	Leads to embryo abortion	Lurin et al. (2013)

Table A7. Candidate genes associated with the QTL related to different traits under heat stress condition (continued).

Sterile/ aborted pods	Chromosome	start	end	Gene annotation	Gene function	References
GSBRNA2T00023864001	chrC05	38,026,436	38,031,421	beta galactosidase 1	Carbohydrate metabolism, pollen development	Sharma et al. (2014)
GSBRNA2T00075666001	chrC05	42,331,911	42,332,659	F-box and associated interaction domains-containing protein	Involved in floral organ development	Jain et al. (2007)
GSBRNA2T00075665001	chrC05	42,332,820	42,333,256	F-box and associated interaction domains-containing protein	Involved in floral organ development	Jain et al. (2007)
GSBRNA2T00075662001	chrC05	42,338,193	42,340,293	Homeodomain-like superfamily protein	Regulate anther dehiscence	Wilson et al. (2011)
GSBRNA2T00075659001	chrC05	42,343,619	42,349,146	SET-domain containing protein lysine methyltransferase family protein	cause embryo abortion	Pontvianne et al. (2010)
GSBRNA2T00075634001	chrC05	42,492,531	42,495,263	apyrase 1	Male transmission completely blocked	Steinebrunner et al. (2003)
GSBRNA2T00075629001	chrC05	42,507,505	42,509,224	Pyruvate kinase family protein	related to early embryo abortion	Zhang et al. (2014)
GSBRNA2T00128854001	chrC07	27,205,457	27,206,291	indole-3-acetic acid inducible 32	induce abortion of flowers	Nico et al. (2015)
GSBRNA2T00128855001	chrC07	27,206,397	27,208,828	Leucine-rich repeat protein kinase family protein	Involved in abnormal anther development	Jia et al. (2008)
GSBRNA2T00128889001	chrC07	27,363,136	27,367,597	homeobox-leucine zipper protein 17	Associated with flowering	Rueda et al. (2005)
GSBRNA2T00019534001	chrUnn_random	6,778,955	6,779,833	F-box/RNI-like/FBD-like domains-containing protein	Involved in floral organ development	Jain et al. (2007)
Pods on main raceme	**Chromosome**	**start**	**end**	**Gene annotation**	**Gene function**	**References**
GSBRNA2T00036883001	chrA02	5,618,950	5,621,701	MATE efflux family protein	Involved in embryo abortion	Zhao et al. (2015)
GSBRNA2T00066329001	chrA05	14,923,082	14,929,060	MATE efflux family protein	Embryo abortion	Zhao et al. (2015)
GSBRNA2T00066327001	chrA05	14,957,816	14,961,501	P450 reductase 1	with reduced number of elongated siliques	Bak et al. (2011)

Table A7. Candidate genes associated with the QTL related to different traits under heat stress condition (continued).

Pods on main raceme	Chromosome	start	end	Gene annotation	Gene function	References
GSBRNA2T00066308001	chrA05	15,071,152	15,072,722	Pentatricopeptide repeat (PPR-like) superfamily protein	Leads to embryo abortion	Lurin et al. (2013)
GSBRNA2T00066307001	chrA05	15,080,249	15,082,335	F-box/RNI-like/FBD-like domains-containing protein	Involved in floral organ development	Jain et al. (2007)
GSBRNA2T00066304001	chrA05	15,096,996	15,099,069	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	Involved with increased pod dehiscence creating oxidative stress	Leisner et al. (2014)
GSBRNA2T00144500001	chrA06	20,857,138	20,858,439	calmodulin-binding protein-related	Involved in embryo development	Radchuk et al. (2006)
GSBRNA2T00144502001	chrA06	20,861,772	20,863,986	Pentatricopeptide repeat (PPR) superfamily protein	Leads to embryo abortion	Lurin et al. (2013)
GSBRNA2T00144513001	chrA06	20,888,721	20,891,712	SNF1 kinase homolog 11	Involved in stress signalgoldmang	Halford et al. (2009)
GSBRNA2T00144518001	chrA06	20,898,100	20,900,195	pectin methylesterase 31	Involved in pollen tube growth	Zhao et al. (2015)
GSBRNA2T00144521001	chrA06	20,903,890	20,905,358	NAC domain containing protein 3	Associated with stress response	Jin et al. (2013)
GSBRNA2T00144548001	chrA06	21,052,298	21,052,848	basic helix-loop-helix (bHLH) DNA-binding family protein	Development and dehiscence of the seed and pod	Hudson et al. (2015)
GSBRNA2T00150214001	chrA10	1,583,137	1,584,495	translocon at the inner envelope membrane of chloroplasts 20	tapetal function and microspore development in *Brassica*	Dun et al. (2011)
GSBRNA2T00086284001	chrA10	1,638,312	1,642,124	glutamate receptor 3.4	Oxidative stress, and B-defficiency	Bargaz et al. (2015)
GSBRNA2T00086287001	chrA10	1,644,665	1,650,390	homeodomain GLABROUS 2	Regulate anther dehiscence	Wilson et al. (2011)
GSBRNA2T00135727001	chrA10	14,312,130	14,314,781	RAB geranylgeranyl transferase beta subunit 1	essential for male fertility	Gutkowska et al. (2014)
GSBRNA2T00135730001	chrA10	14,319,545	14,321,924	calcium-dependent protein kinase 17	Involved in pollen tube tip growth	Myers et al. (2009)

Table A7. Candidate genes associated with the QTL related to different traits under heat stress condition (continued).

Pods on main raceme	**Chromosome**	**start**	**end**	**Gene annotation**	**Gene function**	**References**
GSBRNA2T00135738001	chrA10	14,338,348	14,340,789	pentatricopeptide (PPR) repeat-containing protein	Leads to embryo abortion	Lurin et al. (2013)
GSBRNA2T00135742001	chrA10	14,355,102	14,355,566	17.6 kDa class II heat shock protein	Involved in heat stress tolerant	Al-Whaibi. (2010)
GSBRNA2T00135764001	chrA10	14,419,789	14,420,289	Plant self-incompatibility protein S1 family	severely reduced pollen coats and male sterility	Samuel et al. (2009)
GSBRNA2T00135765001	chrA10	14,420,543	14,421,879	K+ efflux antiporter 6	Associated with pollen tube development and fertilization	Lu et al. (2011)
GSBRNA2T00123633001	chrC03	23,295,425	23,304,438	Cysteine/Histidine-rich C1 domain family protein	Involved in Tapetal Programmed Cell Death and pollen grain sterility	Zhang et al. (2014)
GSBRNA2T00123636001	chrC03	23,323,996	23,326,131	calmodulin-binding family protein	Involved in embryo development	Radchuk et al. (2006)
GSBRNA2T00123637001	chrC03	23,327,874	23,329,653	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	Involved with increased pod dehiscence creating oxidative stress	Leisner ea al. (2014)
GSBRNA2T00123644001	chrC03	23,354,234	23,357,932	Protein kinase family protein	pollen abortion in barley	Radchuk et al. (2006)
GSBRNA2T00068140001	chrCnn_random	8,658,749	8,660,163	Zinc finger C-x8-C-x5-C-x3-H type family protein	cellular regulation in plants	Wu et al. (2014)
GSBRNA2T00068144001	chrCnn_random	8,674,490	8,677,292	GDSL-like Lipase/Acylhydrolase superfamily protein	ethylene production, plays an important role in embryo abortion	Zhao et al. (2015)
GSBRNA2T00071888001	chrCnn_random	8,746,117	8,747,363	F-box family protein	Reduced flower fertility	Ariizumi et al. (2011)

Table A8. Genotypes, plant introduction number and collection site/origin of the accession used for the study.

Genotypes	Plant Introduction number	Collection site/origin
NDSU 0472	Not available	USA
NDSU 0474	Not available	USA
NDSU 0620	Not available	USA
NDSU 0728	Not available	USA
NDSU 10999	Not available	USA
NDSU 15989	Not available	USA
NDSU 161013	Not available	USA
NDSU 31011	Not available	USA
NDSU 41000	Not available	USA
NDSU 7997	Not available	USA
NDSU 81000	Not available	Canada
NDSU 91013	Not available	USA
Azuma	PI 469730	South Korea
Bingo	PI 546468	USA
BO-63	Ames 15651	Canada
Bronowski	PI 469737	Poland
Buk Wuk 3	PI 469738	South Korea
Celebra	PI 538766	Sweeden
Ceskia Tabor	Ames 2793	Czechoslovakia
Comet	PI 649130	Sweden
Cougar	Not available	Canada
delta	PI 543937	Sweden
Evvin	PI 633131	Russian Federation
France 1	PI 469791	France
Galant	Not available	Serbia
Galaxy	Ames 15938	Sweden
Gisora	PI 458948	Germany
Global	PI 601200	Sweden
Golden	PI 649126	Canada
Gora	PI 458949	Germany
Gulle	PI 458936	Sweden
Gullivar	PI 458937	Sweden
Gylle	PI 469812	South Korea
Helga	PI 649136	Germany
Hi-Q	Not available	Canada
IR-2	PI 531280	Hungary

Table A8. Genotypes, plant introduction number and collection site/origin of the accession used for the study (continued).

Genotypes	Plant Introduction number	Collection site/origin
Janetzkis	PI 469826	South Korea
Jasna	Not available	Serbia
Kanada	Not available	Poland
Klinki	PI 469840	South Korea
Kosa	PI 458951	Germany
Koubun	PI 469841	South Korea
Kraphhauser	PI 469842	South Korea
Kritmar rape	PI 469843	South Korea
Legend	PI 633118	USA
Lieikoposki	PI 469887	South Korea
Lifura	PI 469888	South Korea
Lisora	PI 458953	Germany
Major	PI 469891	South Korea
Mali	PI 469894	South Korea
Midas	PI 431571	Canada
Miekuro Dane	PI 469901	South Korea
Mlochowski	PI 535848	Poland
Nabo	PI 469944	Korea South
Nilla 1022	PI 469947	South Korea
Nilla glossy	PI 469946	South Korea
NU 51084	PI 633124	Sweden
Oro	PI 458930	Canada
Orpal	PI 458968	France
Polo canola	Ames 26635	USA
Printol	PI 552810	USA
Prota	PI 458955	Germany
Q2	Not available	Canada
Rang	PI 470013	South Korea
Ratnik	Not available	Serbia
Regent	PI 431572	Canada
Regina II	Ames 1669	Canada
Reston	PI 649152	USA
Rico	PI 458956	Germany
Romeo	PI 458971	France
Russia 5	PI 470021	Former Soviet Union
S.V. Gulle	PI 470032	South Korea

Table A8. Genotypes, plant introduction number and collection site/origin of the accession used for the study (continued).

Genotypes	Plant Introduction number	Collection site/origin
Seoul	PI 537090	South Korea
Shang you	PI 391553	China
Silex	Not available	Canada
Sunrise	PI 597352	USA
SVALOF GULLEN	PI 470033	South Korea
Taiwan	PI 470039	Taiwan
Tokiwa	PI 470049	South Korea
Tonus	PI 470050	South Korea
Topas	PI 601201	Sweden
Tower	PI 431574	Canada
Vostochno-sibirskii	PI 633126	Russian Federation
Wasefuji	PI 470054	South Korea
Westar	Ames 26653	Canada
Willa	PI 470058	South Korea
Yonkkaichi kwo	PI 470061	South Korea
Yudal	PI 470065	South Korea

Table A9. List of germplasms and phenotypic mean data of three different traits under heat stress and controlled condition in greenhouse.

		Heat stress			Control		
Genotypes	**Gen. number**	**Total# Pod**	**#Sterile pod**	**Pollen sterility (%)**	**#Total Pod**	**#Sterile pod**	**Pollen sterility (%)**
Azuma	g108	50.50	13.00	91.33	55.00	0.001	1.000
Bingo	g5	44.33	5.333	12.00	35.33	0.001	0.333
BO-63	g235	22.66	15.66	22.00	41.00	2.500	0.001
Bronowski	g28	30.33	14.33	5.667	37.00	0.001	0.001
Buk Wuk 3	g158	42.66	9.333	10.33	38.00	0.001	0.001
Celebra	g310	38.00	14.33	24.33	35.00	0.001	0.001
Ceskia Tabor	g191	32.00	5.000	33.66	36.00	0.001	0.667
Comet	g10	37.00	20.00	5.333	50.00	0.500	0.333
Cougar	g12	20.00	21.66	39.00	35.00	0.001	0.001
Delta	g113	23.00	8.333	11.33	49.66	1.667	0.001
Evvin	g119	41.00	14.33	3.333	41.50	0.001	0.333
France 1	g124	30.33	8.333	35.33	38.33	0.001	0.001
Galant	g25	32.33	14.66	15.33	52.00	1.667	0.001
Galaxy	g127	29.66	9.000	12.00	39.00	0.001	0.333

Table A9. List of germplasms and phenotypic mean data of three different traits under heat stress and controlled condition in greenhouse (continued).

		Heat stress			Control		
Genotypes	**Gen. number**	**Total# Pod**	**#Sterile pod**	**Pollen sterility (%)**	**#Total Pod**	**#Sterile pod**	**Pollen sterility (%)**
Gisora	g27	30.33	18.33	21.00	51.50	2.000	0.001
Global	g345	44.00	17.00	11.33	52.66	1.667	0.667
Golden	g30	25.00	6.000	10.00	46.50	0.001	0.333
Gora	g131	31.33	8.667	56.00	44.00	0.001	1.333
Gulle	g66	30.33	7.333	2.333	41.00	0.001	0.001
Gullivar	g31	42.66	28.33	11.00	36.66	2.000	0.001
Gylle	g32	24.50	15.00	41.66	32.50	0.500	0.333
Helga	g134	24.50	4.500	54.33	33.50	0.001	1.000
Hi-Q	g34	16.33	16.00	8.333	29.33	0.001	0.333
IR-2	g261	31.33	10.00	20.00	19.50	0.500	0.001
Janetzkis	g139	25.33	6.000	8.333	39.00	0.001	0.001
Jasna	g357	36.66	11.33	12.66	49.00	2.333	0.001
Kanada	g43	19.33	7.000	11.33	28.50	1.000	0.667
Klinki	g145	23.00	18.00	10.00	50.00	0.001	0.001
Kosa	g148	29.33	9.333	37.00	23.66	0.001	0.333
Koubun	g149	23.00	16.00	33.33	13.00	0.500	0.667
Kraphhauser	g152	30.00	11.00	32.33	37.50	0.001	0.333
Kritmar rape	g151	29.00	21.50	6.000	24.50	2.000	0.333
Legend	g48	24.33	7.667	93.00	21.50	0.001	0.333
Lieikoposki	g274	31.00	27.00	22.00	29.00	3.500	1.667
Lifura	g53	12.66	10.66	17.33	33.00	0.001	0.001
Lisora	g161	21.50	8.500	3.000	25.00	0.001	0.001
Major	g63	44.50	23.50	11.00	53.00	1.500	0.001
Mali	g163	66.50	32.50	10.33	44.33	5.667	0.001
Midas	g166	22.66	7.000	0.333	23.50	0.001	0.001
Miekuro Dane	g167	18.00	15.50	26.00	21.00	0.001	0.333
Mlochowski	g171	42.50	13.00	22.00	59.00	0.001	1.000
Nabo	g177	22.00	11.66	69.66	28.00	0.001	0.001
NDSU0472	g208	38.33	16.33	34.66	36.00	0.001	0.001
NDSU0474	g210	25.33	21.00	7.333	20.33	0.001	0.001
NDSU0620	g213	32.66	13.00	8.000	21.66	0.001	0.667
NDSU0728	g215	53.66	36.33	11.00	45.66	2.333	0.333
NDSU10999	g217	24.33	18.66	8.000	33.33	2.000	0.001
NDSU15989	g218	23.66	13.33	3.667	26.66	0.333	0.001
NDSU161013	g219	33.33	28.00	17.66	14.66	0.001	1.333
NDSU31011	g220	40.00	20.33	12.66	21.33	0.001	0.667
NDSU41000	g221	29.00	7.000	65.33	21.00	0.001	0.001

Table A9. List of germplasms and phenotypic mean data of three different traits under heat stress and controlled condition in greenhouse (continued).

		Heat stress			Control		
Genotypes	**Gen. number**	**Total# Pod**	**#Sterile pod**	**Pollen sterility (%)**	**#Total Pod**	**#Sterile pod**	**Pollen sterility (%)**
NDSU7997	g222	21.66	11.66	17.00	50.00	1.333	0.001
NDSU81000	g224	20.66	20.00	4.333	46.33	0.001	0.333
NDSU91013	g225	15.33	2.667	8.000	22.66	1.000	0.001
Nilla 1022	g290	44.00	10.00	21.00	45.00	0.001	0.001
Nilla glossy	g179	28.50	9.000	12.00	41.00	0.001	0.001
NU 51084	g299	35.00	5.000	33.00	25.00	0.500	0.001
Oro	g182	22.00	13.00	12.33	28.00	0.001	0.001
Orpal	g183	32.00	9.500	14.33	40.00	0.001	1.333
Polo canola	g184	28.00	12.00	14.33	25.00	1.000	0.001
Printol	g323	25.00	16.00	19.33	33.33	0.667	0.001
Prota	g334	8.000	5.500	93.00	24.00	0.001	0.001
Q2	g72	23.66	14.00	24.00	42.50	0.500	0.001
Rang	g325	38.66	25.66	14.66	37.66	0.001	0.001
Ratnik	g73	26.33	15.00	20.00	31.00	0.001	0.001
Regent	g187	22.00	4.000	15.33	42.00	0.500	2.333
Regina II	g294	25.66	13.66	8.000	26.50	0.001	0.001
Reston	g327	20.50	7.500	39.00	14.00	0.001	1.000
Rico	g339	39.00	6.500	10.33	30.00	2.000	0.001
Romeo	g75	31.00	15.00	11.66	34.50	0.001	0.001
Russia 5	g341	30.00	15.33	9.000	38.50	0.001	0.001
S.V. Gulle	g342	38.00	13.66	24.00	54.50	1.500	0.001
Seoul	g190	23.33	16.66	3.000	23.00	0.001	0.001
Shang you	g212	19.66	19.66	2.333	15.50	0.500	0.001
Silex	g78	18.00	8.500	6.000	33.00	0.001	0.001
Sunrise	g194	37.66	4.667	33.33	28.00	0.001	0.333
Svalof gullen	g297	23.66	19.00	28.00	30.00	0.001	0.001
Taiwan	g80	48.00	4.500	94.33	45.00	0.001	0.001
Tokiwa	g83	9.667	7.667	31.66	15.50	0.001	0.333
Tonus	g302	34.33	21.00	11.00	40.50	0.001	0.001
Topas	g84	36.00	24.00	8.667	38.50	0.001	0.001
Tower	g86	29.00	13.66	1.000	33.00	0.001	0.001
V.-sibirskii	g96	31.33	2.000	1.333	28.66	0.001	0.001
Wasefuji	g307	18.00	8.333	12.00	21.66	0.001	0.001
Westar	g99	25.00	12.33	9.000	17.33	0.333	0.001
Willa	g102	30.00	9.667	0.001	40.50	0.500	0.001
Yon. kwo	g203	59.33	2.000	16.00	74.50	0.001	0.001
Yudal	g205	27.33	12.66	20.33	34.66	0.001	0.333

Table A10. List of germplasms and Heat Susceptibility Index (HSI) of three different traits under controlled condition.

		Heat Susceptibility Index (HSI)		
Genotypes	**Gen. number**	**Total# Pod**	**Sterile pod**	**Pollen sterility**
Azuma	g108	0.614	498.60	1.0380
Bingo	g5	-1.913	204.53	0.4020
BO-63	g235	3.358	0.2020	252.82
Bronowski	g28	1.353	549.74	65.112
Buk Wuk 3	g158	-0.922	357.95	118.74
Celebra	g310	-0.644	549.74	279.63
Ceskia Tabor	g191	0.834	191.74	0.5690
Comet	g10	1.952	1.4960	0.1720
Cougar	g12	3.218	831.02	448.19
delta	g113	4.032	0.1530	130.23
Evvin	g119	0.090	549.74	0.1030
France 1	g124	1.567	319.60	406.05
Galant	g25	2.840	0.2990	176.20
Galaxy	g127	1.797	345.17	0.4020
Gisora	g27	3.086	0.3130	241.32
Global	g345	1.236	0.3530	0.1840
Golden	g30	3.472	230.10	0.3330
Gora	g131	2.162	332.38	0.4710
Gulle	g66	1.954	281.24	26.804
Gullivar	g31	-1.229	0.5050	126.40
Gylle	g32	1.848	1.1120	1.4250
Helga	g134	2.017	172.56	0.6130
Hi-Q	g34	3.328	613.67	0.2760
IR-2	g261	-4.557	0.7290	229.83
Janetzkis	g139	2.631	230.10	95.758
Jasna	g357	1.890	0.1480	145.55
Kanada	g43	2.415	0.2300	0.1840
Klinki	g145	4.055	690.38	114.91
Kosa	g148	-1.798	357.95	1.2640
Koubun	g149	-5.776	1.1890	0.5630
Kraphhauser	g152	1.502	421.88	1.1030
Kritmar rape	g151	-1.379	0.3740	0.1950
Legend	g48	-0.990	294.03	3.1950
Lieikoposki	g274	-0.518	0.2580	0.1400
Lifura	g53	4.627	409.10	199.18
Lisora	g161	1.051	325.99	34.466
Major	g63	1.204	0.5630	126.40
Mali	g163	-3.755	0.1820	118.74

Table A10. List of germplasms and Heat Susceptibility Index (HSI) of three different traits under controlled condition (continued).

		Heat Susceptibility Index (HSI)		
Genotypes	**Gen. number**	**Total# Pod**	**Sterile pod**	**Pollen sterility**
Midas	g166	0.266	268.46	3.8190
Miekuro Dane	g167	1.073	594.49	0.8850
Mlochowski	g171	2.100	498.60	0.2410
Nabo	g177	1.609	447.45	800.62
NDSU0472	g208	-0.487	626.45	398.39
NDSU0474	g210	-1.846	805.45	84.266
NDSU0620	g213	-3.812	498.60	0.1260
NDSU0728	g215	-1.315	0.5590	0.3680
NDSU10999	g217	2.027	0.3200	91.927
NDSU15989	g218	0.845	1.4960	42.127
NDSU161013	g219	-9.557	1073.9	0.1410
NDSU31011	g220	-6.570	779.88	0.2070
NDSU41000	g221	-2.861	268.46	750.82
NDSU7997	g222	4.255	0.2970	195.35
NDSU81000	g224	4.160	767.10	0.1380
NDSU91013	g225	2.429	0.0640	91.927
Nilla 1022	g290	0.167	383.53	241.32
Nilla glossy	g179	2.289	345.17	137.89
NU 51084	g299	-3.004	0.3450	379.23
Oro	g182	1.609	498.60	141.72
Orpal	g183	1.502	364.35	0.1120
Polo canola	g184	-0.901	0.4220	164.71
Printol	g323	1.877	0.8820	222.17
Prota	g334	5.006	210.92	1068.7
Q2	g72	3.328	1.0360	275.80
Rang	g325	-0.199	984.45	168.54
Ratnik	g73	1.130	575.31	229.83
Regent	g187	3.576	0.2680	0.0640
Regina II	g294	0.236	524.17	91.927
Reston	g327	-3.486	287.63	0.4370
Rico	g339	-2.253	0.0860	118.74
Romeo	g75	0.762	575.31	134.06
Russia 5	g341	1.658	588.10	103.42
S.V. Gulle	g342	2.273	0.3110	275.80
Seoul	g190	-0.109	639.24	34.466
Shang you	g212	-2.019	1.4700	26.804
Silex	g78	3.413	325.99	68.943
Sunrise	g194	-2.592	178.96	1.1380
Svalof gullen	g297	1.585	728.74	321.77

Table A10. List of germplasms and Heat Susceptibility Index (HSI) of three different traits under controlled condition (continued).

		Heat Susceptibility Index (HSI)		
Genotypes	**Gen. number**	**Total# Pod**	**Sterile pod**	**Pollen sterility**
Taiwan	g80	-0.501	172.56	1084.0
Tokiwa	g83	2.826	294.03	1.0800
Tonus	g302	1.143	805.45	126.40
Topas	g84	0.488	920.52	99.589
Tower	g86	0.910	524.17	11.481
Vostochno-sibirskii	g96	-0.699	76.675	15.312
Wasefuji	g307	1.271	319.60	137.89
Westar	g99	-3.321	1.3810	103.42
Willa	g102	1.947	0.7030	0.0000
Yonkkaichi kwo	g203	1.529	76.675	183.86
Yudal	g205	1.588	485.81	0.6900

Table A11. ANOVA for the five different traits of *Brassica napus* under field.

Traits	**SV**	**Df**	**SS**	**MS**	**F-value**	**Pr> F**
Plant height	Rep	2	221.633	110.816	1.27	0.2822
	Genotype	84	40133.4	477.778	5.50	<.0001
Raceme height	Rep	2	438.407	219.203	4.47	0.0128
	Genotype	84	18598.0	221.404	4.52	<.0001
No of pods per raceme	Rep	2	158.713	79.3568	1.50	0.2262
	Genotype	84	17090.3	203.456	3.84	<.0001
Pod length	Rep	2	27.6141	13.8070	27.97	<.0001
	Genotype	84	160.022	1.90502	3.86	<.0001
Abortion	Rep	2	26.5613	13.2806	1.89	0.1549
	Genotype	84	950.620	11.3169	1.61	0.0049

Table A12. Correlation among the traits under natural heat stress in the field.

Traits	**Plant ht**	**Raceme ht**	**Pods on main raceme**	**Pod length**
Plant ht	--------			
Raceme ht	0.43***			
Pods on main raceme	0.39***	0.69***		
Pod length	0.0073 ns	0.12 ns	0.044 ns	
Abortion	0.0052 ns	0.06 ns	0.34**	0.023 ns

*, **, and *** = significantly different at 0.05 0.01, and 0.001 levels of probability, respectively; ns=not significant at P=0.05.

Table A13. Statistical summary of significant markers associated with five different traits of *B. napus* under field condition.

Marker	Chr	Pos	*Log 10 P*	R^2 (%)	Allele 1	# Obs	Mean	Allele 2	# Obs	Mean	Het Allele	# Obs	Mean
Plant height													
chrC08_32368215	C08	32368215	2.40E-05	24	A	01	134.0	G	77	96.80	R	07	92.40
chrC03_545192	C03	545192	2.61E-05	24	G	52	94.10	T	18	103.5	K	15	98.50
chrCnn_rand_78509836	Cnn_rand	78509836	2.99E-05	23	C	07	98.40	T	72	95.10	Y	06	116.5
chrAnn_rand_765860	Ann_rand	765860	3.12E-05	24	G	40	96.50	T	02	131.5	K	43	95.70
chrC03_372591	C03	372591	6.60E-05	23	G	49	92.40	0	00	0000	R	36	103.0
chrC07_2726204	C07	2726204	8.22E-05	22	A	04	113.3	G	74	95.50	R	07	102.0
chrA01_7984469	A01	7984469	9.21E-05	22	C	03	113.3	G	25	99.00	S	57	95.10
chrC04_21172310	C04	21172310	9.42E-05	18	A	79	95.80	C	03	120.2	m	03	101.8
chrC06_5174439	C06	5174439	1.00E-04	22	A	05	91.10	G	78	96.40	R	02	129.5
chrA05_20106451	A05	20106451	1.02E-04	22	A	07	98.30	T	77	96.30	W	01	134.0
chrA06_6086476	A06	6086476	1.15E-04	21	C	01	106.0	T	68	98.40	Y	16	89.70
chrC07_38565142	C07	38565142	1.57E-04	21	A	62	98.50	G	13	91.80	R	10	93.70
chrA03_22665672	A03	22665672	1.79E-04	21	A	08	108.8	G	03	100.0	R	74	95.40
chrA05_7059071	A05	7059071	1.87E-04	20	A	02	104.8	G	78	95.80	R	05	111.2
chrA01_2767171	A01	2767171	1.98E-04	20	A	80	97.30	C	04	80.50	m	01	134.0
chrC03_8183959	C03	8183959	2.04E-04	20	C	63	96.40	T	07	115.5	Y	15	90.40
chrCnn_rand_70816687	Cnn_rand	70816687	2.09E-04	20	A	07	108.8	G	55	95.60	R	23	96.40
chrCnn_rand_70816709	Cnn_rand	70816709	2.10E-04	20	A	10	112.6	G	48	93.90	R	27	96.50
chrCnn_rand_70816722	Cnn_rand	70816722	2.10E-04	20	C	15	91.80	T	58	96.70	Y	12	104.2
chrCnn_rand_50738072	Cnn_rand	50738072	2.24E-04	20	A	05	96.70	T	79	96.40	W	01	134.0
chrA10_6853378	A10	6853378	2.47E-04	20	A	02	131.3	G	70	95.60	R	13	98.70
chrC07_29694210	C07	29694210	2.53E-04	19	G	69	95.60	T	02	131.3	K	14	98.40
chrC05_4296983	C05	4296983	2.76E-04	20	A	69	95.60	C	02	131.3	m	14	98.40
chrCnn_rand_67537689	Cnn_rand	67537689	2.79E-04	20	A	04	124.3	G	76	95.00	R	05	104.5
chrA03_15121547	A03	15121547	2.85E-04	19	G	04	80.50	T	80	97.20	K	01	134.0

Table A13. Statistical summary of significant markers associated with five different traits of *B. napus* under field condition (continued).

Marker	Chr	Pos	*Log 10 P*	R^2 (%)	Allele 1	# Obs	Mean	Allele 2	# Obs	Mean	Het Allele	# Obs	Mean
chrCnn_rand_993352	Cnn_rand	993352	2.97E-04	19	A	20	94.90	C	39	99.80	m	26	94.10
chrC07_6805964	C07	6805964	2.97E-04	19	A	14	95.60	C	47	99.60	m	24	92.40
chrC06_9550868	C06	9550868	3.29E-04	19	A	77	96.50	T	06	91.80	W	02	129.5
chrA01_1046271	A01	1046271	3.62E-04	19	A	64	98.90	C	01	95.70	m	20	90.50
chrA03_rand_254503	A03_rand	254503	3.92E-04	19	A	07	92.40	C	75	96.30	m	03	123.2
chrA03_20343861	A03	20343861	4.45E-04	18	A	12	105.5	G	61	96.50	R	12	90.30
chrA04_rand_1171770	A04_rand	1171770	4.55E-04	19	A	75	98.40	G	07	86.30	R	03	85.20
chrC05_31856881	C05	31856881	4.70E-04	18	C	74	95.60	T	09	108.9	Y	02	91.60
chrC09_39788737	C09	39788737	5.01E-04	18	A	07	86.30	G	75	98.40	R	03	85.20
chrC04_47240683	C04	47240683	5.29E-04	18	A	05	91.40	G	78	96.40	R	02	129.5
chrA06_16667786	A06	16667786	5.54E-04	18	C	55	94.90	T	01	134.0	Y	29	99.30
chrA07_15185460	A07	15185460	5.69E-04	17	A	73	94.90	C	03	115.0	m	09	107.0
chrA07_15185534	A07	15185534	5.69E-04	17	A	19	88.90	G	19	99.80	R	47	98.90
Raceme height													
chrC01_15689071	C01	15689071	1.74E-05	22	G	59	39.70	T	20	36.80	K	06	51.70
chrC01_15689086	C01	15689086	5.77E-05	20	C	55	40.00	T	25	37.50	Y	05	50.10
chrA02_1133295	A02	1133295	8.39E-05	20	A	25	37.50	T	55	40.00	W	05	50.10
chrC01_26101660	C01	26101660	1.18E-04	19	A	25	37.50	T	55	40.00	W	05	50.10
chrA10_1216770	A10	1216770	1.19E-04	16	C	67	39.90	G	03	29.80	S	15	41.90
chrC01_rand_397524	C01_rand	397524	1.40E-04	19	A	76	39.20	G	02	29.30	R	07	50.50
chrCnn_rand_53426788	Cnn_rand	53426788	1.77E-04	18	A	73	39.30	C	04	37.60	m	08	46.40
chrC05_39333990	C05	39333990	1.82E-04	18	A	34	36.60	C	04	32.50	m	47	42.80
chrA08_4189934	A08	4189934	1.90E-04	18	A	03	28.40	T	69	41.30	W	13	34.90
chrA10_1216766	A10	1216766	2.70E-04	14	G	34	36.60	T	04	32.40	K	47	42.90
chrC03_5318108	C03	5318108	3.13E-04	17	A	77	39.40	G	02	25.00	R	06	50.30
chrC03_5318113	C03	5318113	3.13E-04	17	A	58	37.60	G	22	43.30	R	05	51.40

Table A13. Statistical summary of significant markers associated with five different traits of *B. napus* under field condition (continued).

Marker	Chr	Pos	*Log 10 P*	R^2 (%)	Allele 1	# Obs	Mean	Allele 2	# Obs	Mean	Het Allele	# Obs	Mean
chrC03_5318131	C03	5318131	3.13E-04	17	C	22	43.30	T	58	37.60	Y	05	51.40
chrC06_5134034	C06	5134034	3.30E-04	17	C	02	51.20	G	43	42.60	S	40	36.40
chrC05_39333995	C05	39333995	3.49E-04	17	G	01	15.50	T	47	40.80	K	37	39.30
chrA03_19993874	A03	19993874	3.73E-04	17	A	12	40.60	T	71	40.20	W	02	25.00
chrCnn_rand_67444895	Cnn_rand	67444895	3.85E-04	17	A	07	51.90	G	46	38.70	R	32	38.90
chrC03_45955764	C03	45955764	4.00E-04	17	C	02	29.30	T	66	39.80	Y	17	41.20
chrC05_1570548	C05	1570548	4.07E-04	17	C	02	29.30	T	78	39.60	Y	05	48.10
chrC09_13311085	C09	13311085	4.25E-04	17	C	16	34.10	T	60	40.00	Y	09	48.90
chrA08_9665215	A08	9665215	4.53E-04	17	A	77	40.20	C	05	37.20	m	03	36.00
chrC03_5316671	C03	5316671	4.55E-04	16	G	46	38.20	T	28	39.50	K	11	47.70
chrA07_14328514	A07	14328514	4.60E-04	16	A	44	38.00	T	33	40.80	W	08	46.30
chrCnn_rand_22215315	Cnn_rand	22215315	5.10E-04	16	G	17	36.30	T	65	40.90	K	03	36.80
chrA04_18703171	A04	18703171	5.35E-04	16	A	18	37.50	G	64	40.70	R	03	36.80
chrC07_35337162	C07	35337162	5.83E-04	16	C	07	43.60	T	76	39.80	Y	02	29.20
chrC07_35337167	C07	35337167	5.83E-04	16	G	77	40.10	T	06	40.90	K	02	29.30
chrA09_10329126	A09	10329126	6.05E-04	16	G	55	37.80	0	00	0000	K	30	43.60
chrA08_6410089	A08	6410089	6.11E-04	16	T	54	37.70	0	00	0000	Y	31	43.60
chrC06_34432380	C06	34432380	6.75E-04	16	C	12	42.20	T	51	37.10	Y	22	44.90
chrC06_34432382	C06	34432382	6.75E-04	16	C	70	40.00	T	02	29.30	Y	13	40.90
chrC08_16828733	C08	16828733	7.07E-04	16	G	58	40.50	T	03	39.30	K	24	38.50
chrA09_10329146	A09	10329146	7.58E-04	16	A	57	40.20	G	03	39.30	R	25	39.20
chrA04_18419248	A04	18419248	7.68E-04	15	A	34	37.80	G	44	39.80	R	07	49.80
chrA08_9665217	A08	9665217	7.73E-04	15	A	20	37.30	C	57	38.80	m	08	53.80
chrC01_14005097	C01	14005097	7.84E-04	15	C	11	43.70	G	65	38.60	S	09	44.30
Pods on main raceme													
chrA10_rand_2092893	A10_rand	2092893	9.42E-05	21	A	43	27.90	G	28	30.00	R	14	37.00

Table A13. Statistical summary of significant markers associated with five different traits of *B. napus* under field condition (continued).

Marker	Chr	Pos	*Log 10 P*	R^2 (%)	Allele 1	# Obs	Mean	Allele 2	# Obs	Mean	Het Allele	# Obs	Mean
chrA10_rand_2092900	A10_rand	2092900	9.42E-05	21	C	62	30.70	T	12	33.10	Y	11	23.00
chrA09_26370461	A09	26370461	1.27E-04	21	A	71	29.00	T	02	27.00	W	12	37.10
chrAnn_rand_10002128	Ann_rand	10002128	2.98E-04	19	C	02	27.00	G	71	29.00	S	12	37.10
chrAnn_rand_10002131	Ann_rand	10002131	2.98E-04	19	A	11	32.50	G	64	30.80	R	10	22.60
chrC05_8102132	C05	8102132	3.17E-04	19	G	28	33.60	T	13	25.80	K	44	29.10
chrC03_8003052	C03	8003052	3.56E-04	19	A	16	25.90	G	11	39.30	R	58	29.40
chrC05_20590198	C05	20590198	4.01E-04	18	C	14	30.00	T	61	29.30	Y	10	34.70
chrC09_3590238	C09	3590238	4.56E-04	18	A	78	30.00	G	05	22.80	R	02	50.50
chrC09_13198438	C09	13198438	4.72E-04	18	A	32	27.80	C	42	30.00	m	11	37.00
chrAnn_rand_10002158	Ann_rand	10002158	5.31E-04	18	A	44	30.10	G	27	27.10	R	14	35.70
chrCnn_rand_61532934	Cnn_rand	61532934	6.26E-04	17	A	28	27.20	G	43	30.10	R	14	35.70
chrA03_15507989	A03	15507989	6.30E-04	17	A	27	27.10	G	44	30.10	R	14	35.70
chrCnn_rand_1663164	Cnn_rand	1663164	6.41E-04	17	A	28	32.50	G	15	22.80	R	42	31.00
chrA03_15507990	A03	15507990	7.41E-04	17	A	10	29.10	G	17	25.10	R	58	31.70
chrA02_8494949	A02	8494949	7.54E-04	17	C	34	30.50	T	10	38.60	Y	41	27.60
chrC01_3055220	C01	3055220	8.91E-04	17	C	04	30.00	T	73	28.80	Y	08	41.60
chrC04_27753800	C04	27753800	8.97E-04	17	A	49	27.90	T	28	33.30	W	08	31.70
chrC04_27753821	C04	27753821	8.97E-04	17	A	28	33.30	G	49	27.90	R	08	31.70
chrC09_3590304	C09	3590304	8.98E-04	17	C	06	33.00	G	50	31.50	S	29	26.90
chrC09_3590301	C09	3590301	9.38E-04	17	C	50	31.50	T	06	33.00	Y	29	26.90
chrC09_3590341	C09	3590341	9.38E-04	17	C	47	31.60	T	08	32.50	Y	30	27.00
chrC01_9232903	C01	9232903	9.47E-04	17	A	04	31.40	T	61	28.20	W	20	35.60
chrA07_2464161	A07	2464161	9.81E-04	17	C	26	29.60	G	48	28.80	S	11	36.80
chrC08_23407953	C08	23407953	9.86E-04	16	A	55	28.00	G	03	26.90	R	27	34.50
Pod length													
chrC02_33478452	C02	33478452	7.34E-06	26	A	26	6.40	G	47	6.90	R	12	5.87

Table A13. Statistical summary of significant markers associated with five different traits of *B. napus* under field condition (continued).

Marker	Chr	Pos	*Log 10 P*	R^2 (%)	Allele 1	# Obs	Mean	Allele 2	# Obs	Mean	Het Allele	# Obs	Mean
chrC09_43471822	C09	43471822	1.24E-05	25	A	25	6.50	G	44	6.90	R	16	5.97
chrAnn_rand_11544915	Ann_rand	11544915	3.50E-05	23	A	38	6.60	G	39	6.80	R	08	5.62
chrC03_58651519	C03	58651519	3.72E-05	23	A	11	7.00	G	68	6.70	R	06	5.37
chrCnn_rand_43507482	Cnn_rand	43507482	4.06E-05	22	C	69	6.70	T	10	7.00	Y	06	5.37
chrA01_23211171	A01	23211171	5.48E-05	22	A	01	4.30	G	70	6.70	R	14	6.21
chrA05_20319571	A05	20319571	6.24E-05	22	C	02	6.90	T	69	6.80	Y	14	5.75
chrA05_20319586	A05	20319586	6.24E-05	22	C	58	6.80	T	22	6.10	Y	05	6.59
chrC07_40163429	C07	40163429	6.27E-05	22	A	09	6.80	C	43	6.90	m	33	6.14
chrC07_40163415	C07	40163415	8.82E-05	21	A	31	6.80	T	22	6.10	W	32	6.80
chrA05_20248573	A05	20248573	9.87E-05	21	A	07	7.40	G	68	6.60	R	10	5.97
chrA05_20360257	A05	20360257	1.10E-04	20	G	28	6.80	T	23	6.86	K	34	6.30
chrC09_43485851	C09	43485851	1.20E-04	20	C	12	5.90	G	64	6.70	S	09	6.76
chrC03_rand_6258549	C03_rand	6258549	1.32E-04	20	A	22	6.70	C	57	6.70	m	06	5.40
chrA10_16471895	A10	16471895	1.42E-04	20	C	74	6.70	T	07	6.80	Y	04	5.25
chrA09_32428648	A09	32428648	1.61E-04	20	A	11	7.20	C	60	6.70	m	14	5.98
chrC01_14825053	C01	14825053	1.74E-04	19	C	21	6.90	T	45	6.80	Y	19	6.00
chrC02_12836241	C02	12836241	1.75E-04	19	A	34	6.80	T	37	6.80	W	14	5.77
chrC01_14825092	C01	14825092	1.80E-04	19	G	40	6.90	T	15	6.00	K	30	6.50
chrA01_23210566	A01	23210566	1.85E-04	19	A	37	6.80	G	34	6.80	R	14	5.77
chrC03_1389931	C03	1389931	2.04E-04	19	C	51	6.70	T	26	6.70	Y	08	5.51
chrC06_27373215	C06	27373215	2.77E-04	19	A	49	6.70	G	26	6.80	R	10	5.72
chrCnn_rand_5115828	Cnn_rand	5115828	2.77E-04	19	A	19	6.40	G	55	6.80	R	11	6.07
chrA03_7472879	A03	7472879	2.78E-04	19	G	75	6.70	T	05	7.10	K	05	5.30
chrC02_3679554	C02	3679554	3.15E-04	18	C	06	6.80	T	77	6.70	Y	02	4.31
chrA03_rand_701774	A03_rand	701774	3.23E-04	18	A	02	4.90	T	76	6.60	W	07	7.45
chrC02_rand_3648671	C02_rand	3648671	3.40E-04	18	A	07	6.20	G	73	6.70	R	05	5.44

Table A13. Statistical summary of significant markers associated with five different traits of *B. napus* under field condition (continued).

Marker	Chr	Pos	*Log 10 P*	R^2 (%)	Allele 1	# Obs	Mean	Allele 2	# Obs	Mean	Het Allele	# Obs	Mean
chrC07_42164245	C07	42164245	3.45E-04	18	C	62	6.70	T	02	4.40	Y	21	6.58
chrC01_16905608	C01	16905608	3.56E-04	18	A	63	6.70	T	02	4.40	W	20	6.60
chrA09_32405078	A09	32405078	3.64E-04	18	A	64	6.50	G	17	7.10	R	04	5.48
chrC02_33559449	C02	33559449	4.00E-04	18	A	42	6.60	T	02	4.30	W	41	6.72
chrA08_11770647	A08	11770647	4.26E-04	18	A	59	6.70	T	20	6.90	W	06	5.21
chrA09_28427974	A09	28427974	4.33E-04	18	C	57	6.70	T	20	6.90	Y	08	5.68
chrC03_12372983	C03	12372983	4.35E-04	18	G	68	6.70	T	14	6.60	K	03	4.90
chrC03_12372984	C03	12372984	4.35E-04	18	C	52	6.70	T	19	7.00	Y	14	5.89
chrA03_4124353	A03	4124353	5.00E-04	17	A	55	6.70	C	22	6.80	m	08	5.62
chrA05_19555932	A05	19555932	5.11E-04	17	G	55	6.70	T	22	6.80	K	08	5.60
chrA04_rand_325342	A04_rand	325342	5.18E-04	17	C	72	6.70	T	08	6.90	Y	05	5.23
Pod Abortion													
chrA03_4072206	A03	4072206	5.20E-06	27	A	09	14.9	T	40	10.1	W	36	8.06
chrC02_13281695	C02	13281695	9.16E-06	26	A	16	7.70	G	20	13.5	R	49	8.90
chrC02_13209276	C02	13209276	2.22E-05	23	A	70	8.90	C	04	9.00	m	11	15.6
chrC02_13209244	C02	13209244	2.22E-05	23	C	04	9.00	T	70	8.90	Y	11	15.6
chrC02_13271272	C02	13271272	2.57E-05	23	C	48	8.50	G	29	12.6	S	08	7.31
chrA03_25984973	A03	25984973	2.70E-05	24	A	51	8.40	G	30	12.5	R	04	6.28
chrC04_5062497	C04	5062497	3.18E-05	24	C	33	12.1	G	36	7.80	S	16	9.42
chrC04_5062481	C04	5062481	3.18E-05	24	G	66	10.9	0	00	000	K	19	5.90
chrC02_13184955	C02	13184955	5.11E-05	22	C	43	9.20	T	07	14.6	Y	35	9.42
chrA07_1117639	A07	1117639	5.74E-05	23	A	76	9.20	G	03	12.3	R	06	14.9
chrA09_31926968	A09	31926968	6.27E-05	22	A	76	9.20	G	03	12.3	R	06	14.9
chrC02_10389605	C02	10389605	8.71E-05	18	C	03	22.9	T	69	9.8	Y	13	6.41
chrA05_22801086	A05	22801086	1.08E-04	22	C	07	5.90	T	64	10.9	Y	14	6.25
chrAnn_rand_19954418	Ann_rand	19954418	1.24E-04	21	C	39	9.90	T	09	16.2	Y	37	8.05

Table A13. Statistical summary of significant markers associated with five different traits of *B. napus* under field condition (continued).

Marker	**Chr**	**Pos**	***Log $_{10}$ P***	**R^2 (%)**	**Allele 1**	**# Obs**	**Mean**	**Allele 2**	**# Obs**	**Mean**	**Het Allele**	**# Obs**	**Mean**
chrC02_13281718	C02	13281718	1.29E-04	21	A	17	7.40	G	45	11.5	R	23	8.12
chrC04_18664330	C04	18664330	1.32E-04	20	A	46	11.5	G	17	7.6	R	22	7.88
chrC03_53189663	C03	53189663	1.66E-04	20	C	47	9.30	T	08	3.2	Y	30	12.1
chrC04_rand_988002	C04_rnad	988002	1.85E-04	20	A	06	3.70	T	72	10.2	W	07	10.0
chrA05_22800912	A05	22800912	2.35E-04	19	A	02	16.7	G	77	9.3	R	06	13.3
chrCnn_rand_40497348	Cnn_rand	40497348	2.38E-04	19	A	06	16.8	G	37	10.5	R	42	8.10
chrCnn_rand_40497358	Cnn_rand	40497358	2.38E-04	19	G	05	4.00	T	80	10.1	0	00	000
chrC03_10545577	C03	10545577	2.51E-04	20	C	05	4.00	T	80	10.1	0	00	000
chrC05_22964001	C05	22964001	2.65E-04	16	C	80	10.1	T	05	4.0	0	00	000
chrC02_30590926	C02	30590926	2.87E-04	20	C	76	9.20	T	02	20.9	Y	07	12.0
chrA10_506637	A10	506637	3.07E-04	16	A	71	9.50	G	03	20.8	R	11	8.38
chrA10_506670	A10	506670	3.07E-04	16	T	50	11.1	0	00	00	Y	35	7.90
chrA10_506671	A10	506671	3.07E-04	16	A	05	18.9	G	76	9.2	R	04	7.87
chrC02_16620103	C02	16620103	3.07E-04	19	C	10	15.1	G	69	9.1	S	06	7.99
chrA02_18851161	A02	18851161	3.13E-04	19	A	69	9.10	G	10	15.1	R	06	7.99
chrCnn_rand_5438858	Cnn_rand	5438858	3.15E-04	19	A	70	9.10	G	10	15.1	R	05	7.82
chrC04_5456736	C04	5456736	3.70E-04	19	A	06	18.6	G	70	9.0	R	09	9.48
chrA07_758005	A07	758005	3.74E-04	18	C	71	9.00	G	06	17.3	S	08	10.7
chrA01_5035031	A01	5035031	3.79E-04	19	A	54	9.80	G	14	12.8	R	17	7.11
chrC08_19108482	C08	19108482	4.16E-04	19	A	61	9.90	G	08	15.6	R	16	6.33
chrC04_5469752	C04	5469752	4.17E-04	19	A	50	11.2	G	04	6.0	R	31	7.89

Table A14. List of candidate genes and their functions associated with the identified QTL for five different traits of *B. napus* under natural heat stress. Gene annotation and functions are described using TAIR 10 database.

Gene model	Chromosome_marker	Gene start	Gene end	Dist. From gene (kb)	Gene annotation	Gene function	References
Plant Height							
BnaA01g05900D	chrA01_2767171	2737963	2740864	29	Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein	involved in plant development by guiding the cleavage of miRNAs	Lin et al. (2015)
BnaC03g01080D	chrC03_545192	495332	497922	50	C2H2-like zinc finger protein	Pay important roles in plant growth and development	Chrispeels HE et al (2000)
BnaC03g01240D	chrC03_545192	560518	561348	15	C2H2-type zinc finger family protein	Play important roles in plant growth and development	Chrispeels HE et al (2000)
BnaC03g01090D	chrC03_545192	499303	500034	46	FASCICLIN-like arabinogalactan-protein 11	Involved in plant growth, development and response to abiotic stress	Zang et al. (2015)
BnaC03g01200D	chrC03_545192	541294	543191	4.0	Heavy metal transport/detoxification superfamily protein	Associated with plant growth and development	Hall (2002)
BnaC03g01300D	chrC03_545192	576852	580223	32	iron regulated 2	Associated with Iron (Fe) availability for plants which is an essential mineral element for plant growth and development	Yang et al. 2013
BnaC03g00970D	chrC03_545192	455153	460800	90	ubiquitin-protein ligase 4	Involved in several biological processes including hormonal control of vegetative growth	Mazzucotelli et al. (2006)
BnaC06g04590D	chrC06_5174439	5223860	5229566	49	ABC-2 type transporter family protein	Involved in plant growth, development and response to abiotic stresses.	Kang et al. (2011)
BnaC07g36580D	chrC07_38565142	38592380	38596801	27	amino acid transporter 1	Support plant growth and development.	Ortiz-Lopez et al. (1999)

Table A14. List of candidate genes and their functions associated with the identified QTL for five different traits of *B. napus* under natural heat stress. Gene annotation and functions are described using TAIR 10 database (continued).

Gene model	Chromosome_marker	Gene start	Gene end	Dist. From gene (kb)	Gene annotation	Gene function	References
BnaC07g36450D	chrC07_38565142	38548562	38549495	17	ethylene-responsive nuclear protein / ethylene-regulated nuclear protein (ERT2)	Regulates plant growth and development through cell elongation, cell division etc	Sakai et al. (1998)
BnaC07g36460D	chrC07_38565142	38549584	38549897	16	ethylene-responsive nuclear protein / ethylene-regulated nuclear protein (ERT2)	Regulates plant growth and development through cell elongation, cell division etc	Sakai et al. (1998)
BnaC07g36630D	chrC07_38565142	38614676	38617462	50	gibberellin 2-oxidase 8	Regulate plant growth	Fang Lo et al. (2008)
BnaC07g36370D	chrC07_38565142	38507328	38509045	58	LSD1 zinc finger family protein	Associated with abiotic stress response to help plant growth	Guan et al. (2016)
BnaC07g04420D	chrC07_6805964	6889281	6889526	83	callose synthase 5	Involved in Cell wall development in plants	Maeda et al. (2014)
BnaC08g34260D	chrC08_32368215	32380223	32380891	12	H(+)-ATPase 2	Involved in plant growth and development	Schubert (1997)
Raceme height							
BnaA02g02460D	chrA02_1133295	1084194	1086329	49	Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein	Involved in plant development by guiding the cleavage of miRNAs	Lin et al. (2015)
BnaA02g02600D	chrA02_1133295	1163071	1164324	30	Plant calmodulin-binding protein-related	Associated with Ca2+ binding and plant growth	Ranty et al. (2006)
BnaA03g40070D	chrA03_19993874	20016598	20017965	23	Plant calmodulin-binding protein-related	Associated with Ca2+ binding and plant growth	Ranty et al. (2006)
BnaA10g02380D	chrA10_1216770	1225987	1227645	9.0	indoleacetic acid-induced protein 10	Enhance plant growth under drought stress condition	Yasin Ashraf et al. (2006)
BnaA10g02280D	chrA10_1216770	1182962	1184260	34	Protein kinase family protein	Involved in stem elongation and vascular development	Matschi et al. (2013)

Table A14. List of candidate genes and their functions associated with the identified QTL for five different traits of *B. napus* under natural heat stress. Gene annotation and functions are described using TAIR 10 database (continued).

Gene model	Chromosome_marker	Gene start	Gene end	Dist. From gene (kb)	Gene annotation	Gene function	References
BnaC01g28340D	chrC01_26101660	26015923	26019679	86	auxin response factor 1	Regulates plant growth and development	Li et al. (2016)
BnaC05g03130D	chrC05_1570548	1498282	1499295	72	mitogen-activated protein kinase kinase kinase 18	Act as signal transporter for cell division and plant growth.	Krishna Sinha et al. (2011)
BnaC05g41640D	chrC05_39333990	39284968	39287221	49	AP2/B3-like transcriptional factor family protein	Play a crucial role in plant growth	Song et al. (2013)
BnaC07g31130D	chrC07_35337162	35326546	35327913	11	Plant calmodulin-binding protein-related	Associated with Ca2+ binding and plant growth	Ranty et al. (2006)
BnaC08g11450D	chrC08_16828733	16841218	16843655	12	H(+)-ATPase 5	Involved in cell growth and development through energy supply	Schubert (1997)
BnaCnng23850D	chrCnn_rand_22215315	22294422	22296810	79	glutamine synthase clone F11	Involved in Cell wall development in plants	Maeda et al. (2014)
BnaCnng67880D	chrCnn_rand_67444895	67522761	67524034	78	ACC oxidase 1	Favoring plant growth and lowering stress susceptibility	Van de poel. (2014)
BnaCnng67860D	chrCnn_rand_67444895	67517034	67517342	72	AP2/B3-like transcriptional factor family protein	Play a crucial role in plant growth	Song et al. (2013)
BnaCnng67850D	chrCnn_rand_67444895	67511585	67513221	67	Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein	Involved in plant development by guiding the cleavage of miRNAs	Lin et al. (2015)
Pods on main raceme							
BnaA09g36310D	chrA09_26370461	26301650	26302519	69	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	Development and dehiscence of the seed and pod	Hudson et al. (2015)
BnaA09g36320D	chrA09_26370461	26309405	26310301	61	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	Development and dehiscence of the seed and pod	Hudson et al. (2015)

Table A14. List of candidate genes and their functions associated with the identified QTL for five different traits of *B. napus* under natural heat stress. Gene annotation and functions are described using TAIR 10 database (continued).

Gene model	Chromosome_marker	Gene start	Gene end	Dist. From gene (kb)	Gene annotation	Gene function	References
BnaA09g36330D	chrA09_26370461	26316121	26317596	54	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	Development and dehiscence of the seed and pod	Hudson et al. (2015)
BnaA09g36490D	chrA09_26370461	26377171	26378577	7.0	Protein kinase superfamily protein	Involved in pollen abortion in barley	Radchuk et al. (2006)
BnaA09g36270D	chrA09_26370461	26279973	26281656	90	Pyruvate kinase family protein	Associated with early embryo abortion	Zhang et al. (2014)
BnaA10g30330D	chrA10_rand_2092900	2167663	2172059	75	ARM repeat superfamily protein	Self-incompatible and reduced pod number	Sharma et al. (2016)
BnaA10g30160D	chrA10_rand_2092900	2096464	2097400	4.0	Chaperone DnaJ-domain superfamily protein	Involved in male sterility to reduce pod number	Yang et al. (2009)
BnaA10g30380D	chrA10_rand_2092900	2185705	2187109	93	DNAJ heat shock N-terminal domain-containing protein	heat shock protein make tolerance to heat and prevent fruit drop	Zhao-Xia Ma et al. (2015)
BnaA10g30100D	chrA10_rand_2092900	2067086	2071217	26	Heat shock protein DnaJ with tetratricopeptide repeat	Act as heat shock protein to reduce pod shedding	Zhao-Xia Ma et al. (2015)
BnaA10g30260D	chrA10_rand_2092900	2128409	2129697	36	NAC domain containing protein 80	Associated with stress response to maintain pod number	Jin et al. (2013)
BnaA10g30030D	chrA10_rand_2092900	2031769	2031999	61	proline-rich family protein	Associated with flower and pod development	Girno et al. (2013)
BnaA10g30230D	chrA10_rand_2092900	2114701	2115945	22	Protein kinase superfamily protein	Involved in pollen abortion in barley	Radchuk et al. (2006)
BnaAnng09390D	chrAnn_rand_10002128	9962281	9963649	40	Adenine nucleotide alpha hydrolases-like superfamily protein	Involved in male sterility	Mok et al. (2001)
BnaAnng09400D	chrAnn_rand_10002128	9968226	9969594	34	Putative endonuclease or glycosyl hydrolase	related to early embryo abortion	Zhang et al. (2014)
BnaC01g05660D	chrC01_3055220	3031434	3035679	24	Homeodomain-like protein	Regulate anther dehiscence	Wilson et al (2011)
BnaC01g05710D	chrC01_3055220	3049542	3052961	6.0	Protein kinase superfamily protein	Involved in pollen abortion in barley	Radchuk et al. (2006)

Table A14. List of candidate genes and their functions associated with the identified QTL for five different traits of *B. napus* under natural heat stress. Gene annotation and functions are described using TAIR 10 database (continued).

Gene model	Chromosome_marker	Gene start	Gene end	Dist. From gene (kb)	Gene annotation	Gene function	References
BnaC01g05800D	chrC01_3055220	3076562	3079532	21	Protein kinase superfamily protein	Involved in pollen abortion in barley	Radchuk et al. (2006)
BnaC01g14090D	chrC01_9232903	9284682	9286256	52	cytochrome P450, family 706, subfamily A, polypeptide 1	Involved in pollen tube development and fertilization	Zhao et al. (2015)
BnaC01g14110D	chrC01_9232903	9294948	9297353	62	Leucine-rich repeat protein kinase family protein	Involved in abnormal anther development	Jia et al.(2008)
BnaC03g15870D	chrC03_8003052	8002560	8004660	0	Protein kinase superfamily protein	Involved in pollen abortion in barley	Radchuk et al. (2006)
BnaC09g06050D	chrC09_3590341	3680596	3681937	90	Pyruvate kinase family protein	Associated with early embryo abortion	Zhang et al. (2014)
BnaC09g06060D	chrC09_3590341	3681993	3682974	92	Pyruvate kinase family protein	Associated with early embryo abortion	Zhang et al. (2014)
Pod length							
BnaA03g09410D	chrA03_4124353	4221654	4223252	97	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily	Involved with increased pod dehiscence creating oxidative stress	Leisner ea al. (2014)
BnaA03g09160D	chrA03_4124353	4125191	4127377	1.0	Cysteine/Histidine-rich C1 domain family protein	Involved in Tapetal Programmed Cell Death and pollen grain sterility	Zhang et al. (2014)
BnaA03g09300D	chrA03_4124353	4182313	4182795	58	heat shock protein 18.2	Associated with male sterility	Kim et al. (2001)
BnaA03g09030D	chrA03_4124353	4074792	4077497	50	zinc finger (C3HC4-type RING finger) family protein	Cellular regulation in plants	Wu et al. (2014)
BnaA03g09400D	chrA03_4124353	4209494	4210678	85	zinc finger (C3HC4-type RING finger) family protein	Cellular regulation in plants	Wu et al. (2014)
BnaA05g28600D	chrA05_20319571	20279526	20281705	40	cellulose synthase like A14	Associated young seedpod development	Park et al. (2013)
BnaA05g28670D	chrA05_20319571	20306046	20307716	14	Cysteine/Histidine-rich C1 domain family protein	Involved in Tapetal Programmed Cell Death	Zhang et al. (2014)

Table A14. List of candidate genes and their functions associated with the identified QTL for five different traits of *B. napus* under natural heat stress. Gene annotation and functions are described using TAIR 10 database (continued).

Gene model	Chromosome_marker	Gene start	Gene end	Dist. From gene (kb)	Gene annotation	Gene function	References
BnaA05g28870D	chrA05_20319571	20369400	20372057	50	Homeodomain-like superfamily protein	Associated with anther development	Wilson et al. (2011)
BnaA05g28800D	chrA05_20319571	20346755	20348752	27	syntaxin of plants 71	Involved in pollen tube growth	Sharma et al. (2014)
BnaA09g48540D	chrA09_32405078	32502371	32504041	97	cellulose synthase 5	Associated young seedpod development	Park et al. (2013)
BnaA09g48520D	chrA09_32405078	32496839	32497309	92	Plant self-incompatibility protein S1 family	severely reduced pollen coats and cause male sterility	Samuel et al. (2009)
BnaA09g48530D	chrA09_32405078	32497556	32500147	92	Plant self-incompatibility protein S1 family	severely reduced pollen coats and cause male sterility	Samuel et al. (2009)
BnaA10g25580D	chrA10_16471895	16428223	16429725	44	cytochrome P450, family 77, subfamily A, polypeptide 9	Involved in pollen tube development and fertilization	Zhao et al. (2015)
BnaA10g25830D	chrA10_16471895	16566363	16566758	94	Plant self-incompatibility protein S1 family	severely reduced pollen coats and cause male sterility	Samuel et al. (2009)
BnaA10g25840D	chrA10_16471895	16570300	16571845	98	Plant self-incompatibility protein S1 family	severely reduced pollen coats and cause male sterility	Samuel et al. (2009)
BnaC01g21150D	chrC01_14825053	14802563	14804403	22	ubiquitin family protein	Involved in several biological processes including hormonal control of vegetative growth	Mazzucotelli et al. (2006)
BnaC01g23340D	chrC01_16905608	16998104	17000946	92	Malectin/receptor-like protein kinase family protein	Involved in cell elongation and vascular development	Matschi et al. (2013)
BnaC02g06930D	chrC02_rand_3648671	3698436	3703165	50	cellulose synthase-like D3	Associated young seedpod development	Park et al. (2013)

Table A14. List of candidate genes and their functions associated with the identified QTL for five different traits of *B. napus* under natural heat stress. Gene annotation and functions are described using TAIR 10 database (continued).

Gene model	Chromosome_marker	Gene start	Gene end	Dist. From gene (kb)	Gene annotation	Gene function	References
BnaC02g06690D	chrC02_rand_3648671	3570561	3573201	78	glutamine synthetase 1;4	Involved in B-defficiency and pod development	Bargaz et al. (2015)
BnaC02g06890D	chrC02_rand_3648671	3680400	3681999	32	syntaxin of plants 21	Involved in pollen tube growth	Sharma et al. (2014)
BnaC03g22510D	chrC03_12372984	12432046	12433221	59	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	Development and dehiscence of the seed and pod	Hudson et al. (2015)
BnaC03g02780D	chrC03_1389931	1377638	1378027	12	auxin response factor 19	Regulates plant growth and development	Li et al. (2016)
BnaC07g38990D	chrC07_40163415	40103302	40106755	60	AGAMOUS-like 24	Floral transition as well as flower and pod development	Yu et al. (2002)
BnaC07g38970D	chrC07_40163415	40096372	40100341	67	P450 reductase 1	pollen abortion with reduced number of elongated siliques	Bak et al. (2011)
BnaC09g41640D	chrC09_43471822	43543421	43544193	72	Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein	Involved in plant development by guiding the cleavage of miRNAs	Lin et al. (2015)
BnaC09g41650D	chrC09_43471822	43544268	43545376	72	Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein	Involved in plant development by guiding the cleavage of miRNAs	Lin et al. (2015)
BnaC09g41340D	chrC09_43471822	43390602	43393373	81	glutamine synthetase 1;4	Involved in B-defficiency and pod development	Bargaz et al. (2015)
Pod Abortion							
BnaA05g33780D	chrA05_22801086	22807580	22808846	6.0	Adenine nucleotide alpha hydrolases-like superfamily protein	Involved in male sterility	Mok et al. (2001)
BnaA05g33680D	chrA05_22801086	22736816	22737232	64	arabinogalactan protein 6	Involved in pollen wall development and pollen tube growth	Lin et al. (2014)

Table A14. List of candidate genes and their functions associated with the identified QTL for five different traits of *B. napus* under natural heat stress. Gene annotation and functions are described using TAIR 10 database (continued).

Gene model	Chromosome_marker	Gene start	Gene end	Dist. From gene (kb)	Gene annotation	Gene function	References
BnaA05g33830D	chrA05_22801086	22826424	22828539	25	cyclophilin 38	Associated with drought stress and fruit drop	Kelish et al. (2014)
BnaA05g33960D	chrA05_22801086	22890974	22893289	90	DP-E2F-like protein 3	causes severe defects during ovulation and fertilization	Chi (2010)
BnaA05g33770D	chrA05_22801086	22805746	22807215	5.0	myb domain protein 57	Associated with drought stress respose (tolerance) to reduce abortion	Baldoni et al. (2015)
BnaA05g33660D	chrA05_22801086	22723364	22725252	78	Protein kinase superfamily protein	Involved in pollen abortion in barley	Radchuk et al. (2006)
BnaA05g33820D	chrA05_22801086	22823882	22826375	23	Protein kinase superfamily protein	Involved in pollen abortion in barley	Radchuk et al. (2006)
BnaA07g01350D	chrA07_1117639	1079325	1079800	38	zinc ion binding	Cellular regulation and act as micronutrient to reduce pod abortion	Wu et al. (2014)
BnaC02g17570D	chrC02_13281695	13199749	13200702	82	AGAMOUS-like 15	Associated with embryonic development	Hill (2007)
BnaC02g17610D	chrC02_13281695	13220239	13222779	61	Protein phosphatase 2C family protein	Involved in drought stress and Abscicic acid production	Su et al. (2016)
BnaC02g17580D	chrC02_13281695	13201203	13202136	80	sulfur E2	Associated with heat stress and decrease the number of seed per plant	Muguet et al. (2015)
BnaC04g07270D	chrC04_5456736	5440327	5442725	16	chaperone protein dnaJ-related	Involved in male sterility and cause sterile pod	Yang et al. (2006)
BnaC04g07270D	chrC04_5456736	5440327	5442725	16	chaperone protein dnaJ-related	Involved in male sterility and pod sterility	Yang et al. (2006)
BnaC04g07470D	chrC04_5456736	5513477	5517264	57	Cyclin/Brf1-like TBP-binding protein	Involved in meiotic division and fruit development	Yang et al. (2006)
BnaC04g07470D	chrC04_5456736	5513477	5517264	57	Cyclin/Brf1-like TBP-binding protein	Involved in meiotic division and fruit development	Yang et al. (2006)

Table A14. List of candidate genes and their functions associated with the identified QTL for five different traits of *B. napus* under natural heat stress. Gene annotation and functions are described using TAIR 10 database (continued).

Gene model	Chromosome_marker	Gene start	Gene end	Dist. From gene (kb)	Gene annotation	Gene function	References
BnaC04g07360D	chrC04_5456736	5460518	5460967	4.0	F-box family protein	Reduced flower fertility and reduce pod set	Ariizumi et al. (2011)
BnaC04g07360D	chrC04_5456736	5460518	5460967	4.0	F-box family protein	Reduced flower fertility and reduce pod set	Ariizumi et al. (2011)
BnaC04g01120D	chrC04_rand_988002	912838	913631	75	arabinogalactan protein 16	Involved in pollen wall development and pollen tube growth	Xiao et al. (2014)
BnaC04g01270D	chrC04_rand_988002	1002167	1003856	14	ARID/BRIGHT DNA-binding domain;ELM2 domain protein	Involved in embryonic development	https://en.wikipedia.org/wiki/ARID_domain
BnaC04g01250D	chrC04_rand_988002	984082	987459	4.0	cyclic nucleotide gated channel 3	Involved in meiotic division and fruit development	Yang et al. (2006)
BnaC04g01080D	chrC04_rand_988002	904940	905783	83	cytokinin response factor 5	Prevent flower abortion in Lupin	Song et al. (2015)
BnaC04g01230D	chrC04_rand_988002	971440	972082	17	Homeodomain-like superfamily protein	Regulate anther dehiscence	Wilson et al. (2011)
BnaC04g01390D	chrC04_rand_988002	1062714	1064045	75	NAC domain containing protein 6	Associated with strjiaess response to prevent pod abortion	Jin et al. (2013)
BnaC05g26700D	chrC05_22964001	22907120	22910098	57	ARM repeat superfamily protein	Pollen become self-incompatible and cause pod abortion	Sharma et al. (2016)
BnaC05g26680D	chrC05_22964001	22881981	22883636	82	cytochrome P450, family 705, subfamily A, polypeptide 27	pollen abortion with reduced number of elongated siliques	Bak et al. (2011)

Table A15. Daily weather data of July 3, 2014 to July 23, 2014, during flowering to pod setting stage of canola in this study (https://ndawn.ndsu.nodak.edu).

Fargo					
Date	**Max Air Temp (°F)**	**Min Air Temp (°F)**	**Avg Temp (°F)**	**Diurnal Temp Range (°F)**	**Avg Bare Soil Temp (°F)**
2014-07-03	77	49	63	28	67
2014-07-04	84	63	73	21	68
2014-07-05	95	67	81	28	77
2014-07-06	83	69	76	14	76
2014-07-07	82	61	71	21	72
2014-07-08	75	59	67	16	70
2014-07-09	77	57	67	20	72
2014-07-10	84	59	72	25	71
2014-07-11	87	65	76	22	76
2014-07-12	82	59	70	23	73
2014-07-13	75	53	64	22	69
2014-07-14	68	50	59	19	64
2014-07-15	73	47	60	26	67
2014-07-16	79	51	65	28	72
2014-07-17	82	62	72	20	74
2014-07-18	85	64	75	22	76
2014-07-19	82	61	71	20	77
2014-07-20	94	68	81	26	81
2014-07-21	92	67	80	25	81
2014-07-22	78	61	69	17	75
2014-07-23	79	56	68	22	75
Averages:	**82**	**59**	**70**	**22**	**73**
Max:	**95**	**69**	**81**	**28**	**81**
Min:	**68**	**47**	**59**	**14**	**64**
Std. Dev.:	**7**	**6**	**6**	**4**	**4**

Table A16. Genotypes, plant introduction number and collection site/origin of the accession used for the study.

Genotypes	**Plant Introduction number**	**Collection site/origin**
Aviso	Not available	Canada
Bingo	PI 546468	USA
BO-63	Ames 15651	Canada
Brio	PI 458919	France
Celebra	PI 538766	Sweeden
Colza	PI 469756	South Korea
Comet	PI 649130	Sweden
Conquest	Not available	Canada
Cougar	Not available	Canada
Crystal	PI 601261	Sweden, Malmohus

Table A16. Genotypes, plant introduction number and collection site/origin of the accession used for the study (continued).

Genotypes	Plant Introduction number	Collection site/origin
delta	PI 543937	Sweden
Drakkar	Not available	France
Eckendorfer Mali	PI 469784	South Korea
Evvin	PI 633131	Russian Federation
France 1	PI 469791	France
Galant	Not available	Serbia
Galaxy	Ames 15938	Sweden
Global	PI 601200	Sweden
Golden	PI 649126	Canada
Gora	PI 458949	Germany
Gulle	PI 458936	Sweden
Gullivar	PI 458937	Sweden
Hi-Q	Not available	Canada
IR-2	PI 531280	Hungary
Janetzkis	PI 469826	South Korea
Jasna	Not available	Serbia
Kanada	Not available	Poland
Kosa	PI 458951	Germany
Koubun	PI 469841	South Korea
Legend	PI 633118	USA
Mali	PI 469894	South Korea
Mazowiecki	PI 311730	Polland
Midas	PI 431571	Canada
Miekuro Dane	PI 469901	South Korea
Miekuro Dane	PI 469901	South Korea
Nabo	PI 469944	Korea South
NDSU 0472	Not available	USA
NDSU 0473	Not available	USA
NDSU 0474	Not available	USA
NDSU 0619	Not available	USA
NDSU 0620	Not available	USA
NDSU 0728	Not available	USA
NDSU 0729	Not available	USA
NDSU 1099	Not available	USA
NDSU 151000	Not available	USA
NDSU 15989	Not available	USA
NDSU 161013	Not available	USA
NDSU 31011	Not available	USA

Table A16. Genotypes, plant introduction number and collection site/origin of the accession used for the study (continued).

Genotypes	Plant Introduction number	Collection site/origin
NDSU 41000	Not available	Canada
NDSU 7997	Not available	USA
NDSU0726	Not available	USA
NDSU81000	Not available	USA
NDSU91013	Not available	USA
Nilla 1022	PI 469947	South Korea
NU 41737	PI 649135	Turkey
NU 51084	PI 633124	Sweden
NU 51084	PI 633124	Sweden Malmohus
O 84	PI 478340	Chaina
Oro	PI 458930	Canada
Orpal	PI 458968	France
Polo canola	Ames 26635	USA
Premier	PI 470009	South Korea
Printol	PI 552810	USA
Prota	PI 458955	Germany
Q2	Not available	Canada
Rang	PI 470013	South Korea
Ratnik	Not available	Serbia
Regent	PI 431572	Canada
Regina II	Ames 1669	Canada
Reston	PI 649152	USA
Romeo	PI 458971	France
Russia 5	PI 470021	Former Soviet Union
S.V. Gulle	PI 470032	South Korea
Seoul	PI 537090	South Korea
Silex	Not available	Canada
Sunrise	PI 597352	USA
Svalof gullen	PI 470033	South Korea
Taiwan	PI 470039	Taiwan
Tokiwa	PI 470049	South Korea
Tonus	PI 470050	South Korea
Topas	PI 601201	Sweden
Tower	PI 431574	Canada
Turret	PI 365644	Canada
Vostochno-sibirskii	PI 633126	Russian Federation
Wasefuji	PI 470054	South Korea
Westar	Ames 26653	Canada
Willa	PI 470058	South Korea

Table A17. List of genotypes and their phenotypic mean under field conditions.

Genotypes	Gen. number	plant height (cm)	Raceme height (cm)	Pods on main raceme	Pod length (cm)	Abortion from main raceme	Pollen sterility (%)
Aviso	g366	87.33	39.47	30.67	7.157	7.609	0.330
Bingo	g5	134.0	36.67	27.67	7.397	9.639	0.333
BO-63	g235	98.33	39.73	29.00	6.470	4.598	1.667
Brio	g147	106.0	37.87	27.00	6.900	3.704	0.000
Celebra	g310	92.00	28.00	23.00	6.783	5.797	1.000
Colza	g9	79.00	32.40	18.67	7.407	8.929	1.333
Comet	g10	86.00	36.13	38.67	5.457	8.621	2.000
Conquest	g11	68.00	33.40	31.33	7.623	5.319	36.00
Cougar	g12	86.67	32.40	25.67	6.130	19.48	1.000
Crystal	g13	105.4	60.33	48.33	7.637	6.897	0.660
Czyzowski	g243	131.3	39.00	33.67	7.387	4.950	1.333
Delta	g113	108.3	42.67	37.00	5.970	15.31	2.000
Drakkar	g16	100.0	42.40	27.67	8.210	9.639	0.333
Eckendorfer mali	g247	104.8	54.47	39.67	5.880	1.681	0.660
Evvin	g119	96.00	32.40	17.33	5.587	11.53	0.666
France 1	g124	90.00	36.33	28.33	6.366	7.059	1.660
Galant	g25	103.6	28.13	28.67	5.917	3.488	0.333
Galaxy	g127	88.30	47.33	28.67	7.206	11.62	0.000
Global	g345	95.67	46.33	42.67	6.903	8.594	1.330
Golden	g30	105.0	35.40	27.67	6.297	12.04	0.000
Gora	g131	98.40	55.07	45.33	7.180	2.206	0.660
Gulle	g66	90.00	35.90	24.00	7.407	4.167	0.333
Gulliver	g31	128.6	61.13	52.67	6.807	8.861	1.330
Hi-Q	g34	81.00	41.33	44.00	7.823	11.36	2.667
IR-2	g261	109.0	46.13	29.00	7.235	8.046	0.330
Janetzkis	g139	102.0	38.60	18.33	6.648	14.54	1.000
Jasna	g357	109.0	37.20	25.67	7.650	19.48	0.333
Kanada	g43	95.00	40.93	26.33	7.655	7.595	1.667
Kosa	g148	96.67	30.87	19.67	6.200	16.94	0.333
Koubun	g149	94.33	34.93	32.00	6.323	5.208	1.330
Legend	g48	86.33	33.60	18.33	5.987	1.818	0.000
Mali	g163	92.40	34.27	27.33	6.863	4.878	0.000
Mazowiecki	g316	82.67	26.87	23.00	6.269	15.94	0.000
Midas	g166	97.67	38.67	26.33	6.423	15.19	1.660
Miekuro Dane	g167	88.33	43.00	22.33	7.663	4.478	3.000
Nabo	g177	94.20	36.27	20.67	5.032	3.226	1.333
NDSU0472	g208	104.6	39.80	27.00	7.210	16.04	0.330
NDSU0473	g209	111.0	47.67	37.67	6.603	30.08	0.330
NDSU0474	g210	83.67	15.50	28.67	6.210	23.25	3.000
NDSU0619	g211	75.67	35.20	22.33	5.981	20.89	0.000

Table A17. List of genotypes and their phenotypic mean under field conditions (continued).

Genotypes	Gen. number	plant height (cm)	Raceme height (cm)	Pods on main raceme	Pod length (cm)	Abortion from main raceme	Pollen sterility (%)
NDSU0726	g214	72.10	24.33	13.00	5.936	7.692	1.330
NDSU0728	g215	106.6	53.80	39.33	6.967	8.475	0.660
NDSU0729	g216	106.0	31.00	22.67	7.000	10.29	0.330
NDSU10999	g217	80.67	39.60	36.33	6.887	10.09	1.660
NDSU151000	g105	97.33	45.53	41.33	6.097	6.452	0.000
NDSU15989	g218	86.00	38.67	33.00	7.480	22.22	1.330
NDSU161013	g219	83.00	44.33	36.00	6.103	13.88	0.330
NDSU31011	g220	100.0	37.27	22.33	7.113	16.41	0.330
NDSU41000	g221	99.00	58.60	39.00	7.673	5.983	0.000
NDSU7997	g222	95.33	44.40	36.33	7.490	4.587	1.000
NDSU81000	g224	103.6	37.80	34.33	7.717	6.796	0.330
NDSU91013	g225	93.00	44.80	36.67	6.683	6.364	2.330
Nilla 1022	g290	96.80	45.87	28.00	6.143	5.952	0.333
NU 41737	g318	125.0	44.00	39.67	4.277	6.723	1.330
NU 51084	g299	129.0	51.67	44.33	6.980	4.511	1.000
O 84	g320	105.6	47.20	27.33	4.347	3.659	0.660
Oro	g182	107.0	46.07	29.67	6.530	2.247	0.000
Orpal	g183	95.67	34.87	26.33	7.241	11.39	0.660
Peace	g69	90.67	33.20	22.00	5.370	15.15	0.660
Polo Canola	g184	77.67	29.87	14.33	7.249	9.302	1.333
Premier	g333	94.33	28.27	23.00	7.047	10.14	0.333
Printol	g323	91.33	29.47	22.33	6.343	8.955	1.000
Prota	g334	90.67	34.53	25.33	6.920	6.579	1.660
Q2	g72	95.67	47.33	37.67	6.520	8.850	2.000
Ratnik	g73	98.33	43.80	34.00	5.803	3.922	1.660
Regent	g187	101.6	31.67	27.67	5.993	12.04	2.333
Regina II	g294	108.3	38.20	27.00	7.223	2.469	0.000
Reston	g327	94.33	44.80	38.00	4.900	8.772	0.660
Romeo	g75	100.0	35.00	27.67	6.403	10.84	0.330
Russia 5	g341	94.33	43.27	31.67	6.203	4.211	0.333
Seoul	g190	84.30	56.60	34.33	7.420	8.738	1.000
Silex	g78	102.6	43.00	27.00	6.047	16.04	0.330
Sunrise	g194	81.00	29.87	17.00	6.907	13.72	1.660
Svalof gullen	g297	102.0	42.47	36.00	6.583	10.18	8.667
Taiwan	g80	86.40	38.57	24.00	4.423	8.333	0.330
Tokiwa	g83	72.50	27.53	19.33	6.433	17.24	0.000
Tonus	g302	105.6	49.67	29.67	7.350	8.989	1.330

Table A17. List of genotypes and their phenotypic mean under field conditions (continued).

Genotypes	Gen. number	plant height (cm)	Raceme height (cm)	Pods on main raceme	Pod length (cm)	Abortion from main raceme	Pollen sterility (%)
Topas	g84	108.6	34.20	29.00	6.317	2.299	1.667
Tower	g86	88.00	37.07	31.00	6.773	12.903	2.333
Turret	g303	99.00	46.00	52.33	5.600	20.382	3.667
Vostochno-sibirskii	g96	103.2	49.27	28.33	6.417	10.588	0.330
Wasefuji	g307	108.6	59.47	31.67	6.527	11.579	0.330
Westar	g99	95.67	31.07	32.00	6.953	12.500	1.333
Willa	g102	95.67	40.93	31.67	7.260	11.579	1.330

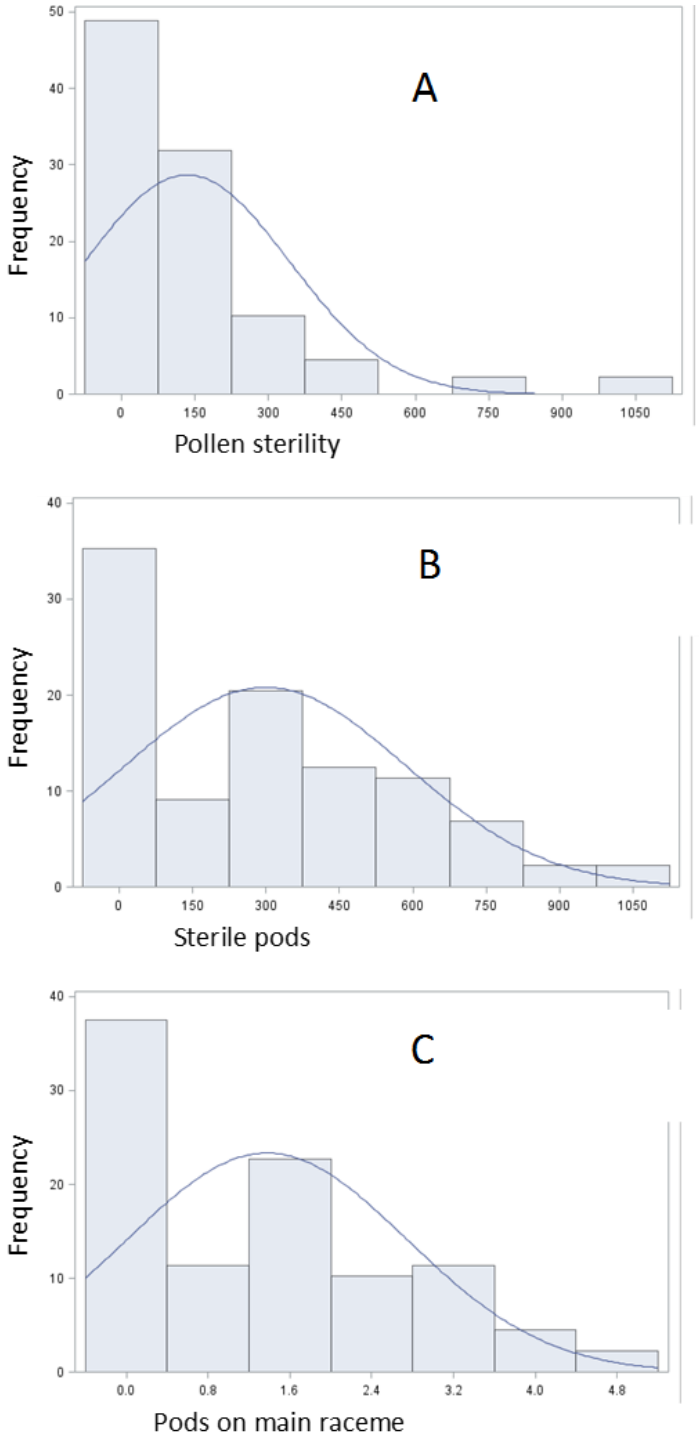

Figure A1. Phenotypic distribution of three different traits under heat stress (A) Pollen sterility (B) Sterile or aborted pods (C) Number of pods on main raceme.

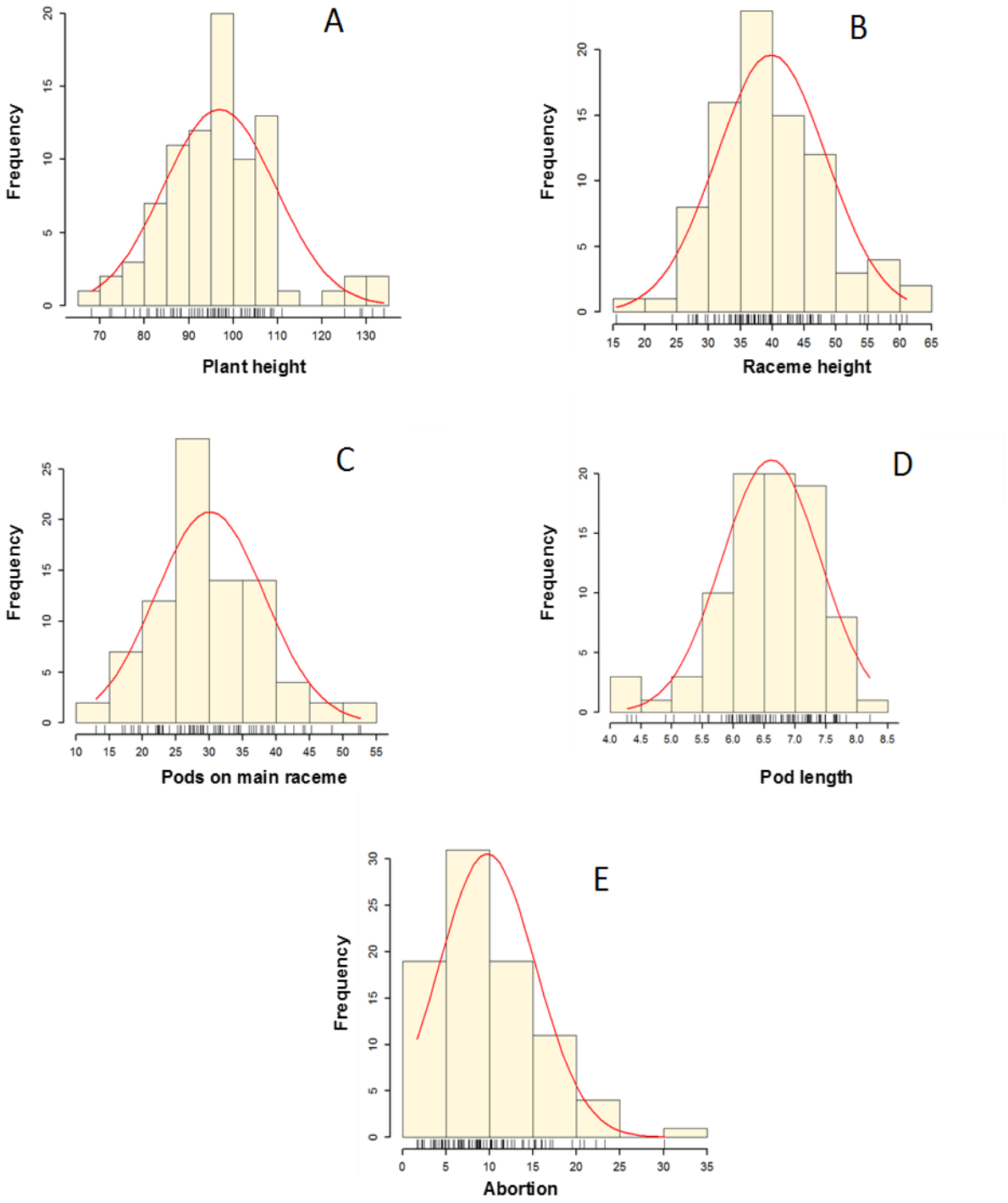

Figure A2. Phenotypic distribution of five different traits under field condition (A) Plant height (cm) (B) Raceme height (cm) (C) Number of pods on main raceme (D) Pod length (cm) (E) Flower and pod abortion.