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Association mapping of agronomic traits of canola (*Brassica napus* L.) subject to heat stress under field conditions

Mizanur Rahaman[1], Sujan Mamidi[2], Mukhlesur Rahman*[1]

[1]Department of Plant Sciences, North Dakota State University, Fargo, ND 58102, USA
[2]HudsonAlpha Institute for Biotechnology, Huntsville, AL, USA

***Corresponding author: md.m.rahman@ndsu.edu**

Abstract

Brassica is a cool season crop and is susceptible to high temperatures. Developing heat stress tolerant varieties will help the crop to sustain under high temperature and can be used to extend the geographical range of cultivation. We have phenotyped 84 spring type *Brassica napus* accessions in field under natural heat conditions. Data on various agronomic traits were collected at the end of flowering to maturity stages. An association mapping study was performed to identify QTL associated with heat stress tolerant agronomic traits. A total of 37,269 single nucleotide polymorphism markers were used for this study. Multiple markers distributed on most of the chromosomes were identified. A total of 6, 11, 7, 11 and 7 QTL were identified those explained 52.2%, 71.8%, 53.2%, 73.5% and 61.0% of the total phenotypic variations for plant height, main raceme height, pods on main raceme, pod length, and sterile/aborted pod, respectively. Multiple candidate genes known to be involved in abiotic stress and abortion of different organs were identified in the vicinity of the QTL. For instance, *B. napus* BnaA03g09160D gene involved in programmed cell death and pollen sterility, BnaA05g33770D and BnaA05g33780D genes associated with pollen sterility and pod abortion were identified in the QTL regions.

Keywords: *Brassica napus*, heat stress, GBS, QTL, association mapping, field study.

Introduction

Rapeseed/canola (*Brassica napus* L.) is an amphidiploid (2n=4x=38, AACC) that originated from the hybridization of two diploid species, *Brassica rapa* (2n=2x=20, AA) and *B. oleracea* (2n=2x=18, CC) (U, 1935; Raymer, 2002). The genome size of this crop is about 1,130 Mb. The C genome is larger than the A genome which is consistent to the genome sizes of *B. oleracea* and *B. rapa*, respectively (Chalhoub et al., 2014). Rapeseed ranks second in the world as an oil-producing crop next to soybean (Foreign Agricultural Service, USDA, October 2016). Within the USA, about 84% of canola is produced in North Dakota with a market value of about $384 million/year (5-year average from 2011-2015; USDA-NASS, January 2016).

Although rapeseed is a valuable oilseed crop, the production of this crop is hampered due to different biotic and abiotic stresses such as disease, pests, heat, drought, cold stress etc. High temperature creates a lethal environment for the growth and development of plants, and produces different types of metabolites, toxins and alters the hormonal activity, which creates abnormal phenotypes. Plants are able to cope with the stress conditions by reducing the growth and development, yield, and by changing morphological, physiological, biochemical, and molecular properties (Bita and Gerats, 2013). Temperature increase of 3-4˚C from its normal range during reproductive stages, even for a short duration, could cause 15-35% yield loss (Ortiz et al., 2008). Generally, the suitable temperature for spring canola production is about 15-20˚C, but the temperature over 27˚C causes pollen sterility and pod abortion (Morrison, 1993; Angadi et al., 2000; Nuttal et al., 1992). Rapeseed production under increased temperature from 28˚C to 35˚C could reduce the seed yield by about 54% to 87% (Gan et al., 2004). It has been estimated that 1˚C temperature increase from the suitable range of crop growth and development in July can cause 10% yield reduction of canola in Saskatchewan, Canada (Nuttal et al., 1992). Heat stress during pre-anthesis stage reduces pollen fertility, whereas post anthesis heat decreases 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). This sensitivity increases the flower abortion, pollen sterility, tapetum degeneration (Oshino et al. 2007; Endo et al., 2009), and reduces the pod development, seed set, assimilatory capacity and productivity (Barnabás et al., 2008), shoot and root growth (Vollenweider and Günthardt-Goerg, 2005), seed yield (Ahuja et al., 2010; Mittler and Blumwald, 2010). The reason of these changes are due to reduced photosynthesis (Zhang et al., 2006), radiation use efficiency (Hasanuzzaman et al., 2013), increased plant respiration (Reynolds et al., 2007), Reactive Oxygen Species (ROS) production (Dat et al., 1998; Gong et al., 1998; Volkov et al., 2006), lipid peroxidation, protein degradation (Savchenko et al., 2002), hyperfluidization and disruption of plant cell membranes (Horváth et al., 1998; Sangwan et al., 2002), metabolic imbalance (Vierling, 1991; Dat et al., 1998; Gong et al., 1998; Volkov et al., 2006), disrupted biosynthesis and compartmentalization of metabolites (Maestri et al., 2002), genomic rearrangements (Ivashuta et al., 2002; Steward et al., 2002), demethylation of transposons (Bennetzen, 2000) and so on.

Heat stress tolerance in plants is a multigenic character. 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 quiet unknown to the scientific community (Shao et al., 2007).

Association mapping (AM) is based on the linkage disequilibrium and utilizes ancestral recombinations and natural genetic diversity within a population to quantify the quantitative traits (Geiringer, 1944; Lewontin and Kojima, 1960), where linkage disequilibrium is a non-random association of alleles at two loci. It is an alternative method to discover genetic factors using biparental crosses, and has a higher mapping resolution within a large number of unrelated individuals. This helps to identify common genetic variants, which control a common phenotype (Risch, 2000). It is relatively new and promising genetic method for complex trait dissection of plants (Zhu et al., 2008), and for QTL identification (Yu et al., 2006). It uses a sample of accessions from the germplasm collections, which have accumulated many rounds of recombination events. This method has been used in many crop and animal species to identify marker-trait associations. As heat stress is a complex trait, AM would be a good approach to locate the genomic regions associated with heat stress affected phenotypes. In the light of these facts, this research scheme has been taken to identify the genomic regions associated with the heat stress traits in a collection of spring type *B. napus* accessions under field conditions.

Results

Phenotyping of plant materials

The phenotypic variation of the five traits were variable in field conditions during the flowering to maturity stages. Of the genotypes, raceme height varied between 15.5 cm and 61.1 cm, and pod/raceme had a range of 13.0 to 52.6. The Shapiro-Wilk test of normality indicated that the population for raceme height ($p < 0.198$) and pod/raceme ($p < 0.150$) are normally distributed (Table 1, Fig. 1). The plant height ranged from 68 cm to 134 cm, pod length ranged between 4.24 cm amd 8.21 cm, and sterile/aborted pod varied between 1.68% and 30.1%. The Shapiro-Wilk test of normality of plant height ($p < 0.008$), pod length ($p < 0.006$), and sterile/aborted pod ($p < 0.0004$) indicated non-normality of the distribution (Table 1, Fig. 1).

Population structure, PCA and relatedness

A total of 37,269 SNP markers were used after removing for minor allele frequency of 5%. About 20.6% heterozygous loci were present in these samples. Principal component analysis has grouped the population into three continuous clusters using the first two principal components (Fig. 2).

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 PC_{17} + kinship was found as the best models for plant height, and pod abortion. The model PC_{17} was the best model for the main raceme height and number of pods on main raceme, whereas PC_3 was the best model for pod length.

During the marker trait association, three markers were found significant for plant height at 0.01 percentile tail of empirical distribution ($p \leq 2.99E-05$, Table 2, Fig. 3). Among these three markers, two were located on chromosomes C03 (0.5 Mbp) and one on C08 (32.368 Mbp). Additionally, 35 markers were found significant at 0.01 percentile tail of the empirical distribution ($p \leq 5.18E-04$; Supplementary table S3). These markers were found on multiple chromosomes. A stepwise regression with these markers identified six significant QTL regions (Table 3, Fig. 4) which together explained 52.2% of the total phenotypic variation. The identified candidate genes associated with this trait include 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), that regulates plant growth and development through cell division, and gibberellin 2-oxidase involved in plant growth and development, were also identified in the QTL regions (Supplementary table S4).

Five markers were significantly associated with raceme height at 0.01 percentile ($p \leq 8.39E-05$; Table 2; Fig. 3). These significant markers were located on chromosome A02 (1.13 Mbp), A10 (1.216 Mbp) and C01 (15.6 and 26.1Mbp). Thirty-one additional markers were found significant at 0.1 percentile tail of the distribution ($p \leq 7.84E-04$, Supplementary table S3). Eleven QTL regions were identified through stepwise regression. These 11 QTL together explained 71.8 % of phenotypic variation and were located on chromosomes A02, A03, A10, C01, C05, C07, and C08 (Table 3, Fig. 4). Many candidate genes such as plant calmodulin-binding protein that is associated with Ca2+ binding, plant growth and development, indole acetic acid-induced protein 10 that enhances plant growth under drought stress condition, protein kinase family protein that is involved in stem elongation and vascular development, ACC oxidase1 that favors plant growth and lowering stress susceptibility were identified (Supplementary table S4).

For number of pods on main raceme, five markers were identified significant at 0.01 percentile ($p \leq 2.98E-04$, Table 2, Fig. 3). One of these markers was located on chromosome A09 (26.3 Mbp). Besides these markers, 20 more markers were found significant at 0.1 percentile tail of the empirical distribution ($p \leq 9.86E-04$, Supplementary table S3). Further, seven major QTL were identified through stepwise regression, which together explained 53.2% of phenotypic variation (Table 3, Fig. 4). Among them, four QTL were located on chromosomes A09, C01, C03 and C09. 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 are present in the QTL regions (Supplementary table S4).

Four markers associated with pod length at 0.01 percentile tail ($p \leq 3.72E-05$, Table 2, Fig. 3) were located on chromosome C02 (33.4 Mbp), C03 (58.6 Mbp) and C09 (43.4 Mbp). Another 34 markers were found significant at 0.1 percentile tail of the empirical distribution ($p \leq 9.87E-05$, Supplementary table S3). A total of 73.5% phenotyping variation was explained by 11 major QTL (Table 3, Fig. 4). These QTL were located on A03, A05, A09, A10, C01, C03, C07, and C09 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 reduce pollen coats and cause male sterility, glutamine synthetase 1:4 which is involved in

Table 1. Variation in different agronomic traits of *B. napus* under natural heat stress in field condition.

Traits	Average	Standard deviation	Maximum	Minimum	p-value of Shapiro-Wilk normality test	Coefficient of Variation
Plant height (cm)	96.9	12.6	134	68.0	0.008	13.0031
Raceme height (cm)	39.9	8.64	61.1	15.5	0.198	21.6541
Pod/raceme	30.2	8.15	52.6	13.0	0.150	26.9868
Pod length (cm)	6.62	0.8	8.21	4.27	0.006	12.0846
Sterile/aborted pod (%)	9.74	5.54	30.1	1.68	0.0004	56.8789

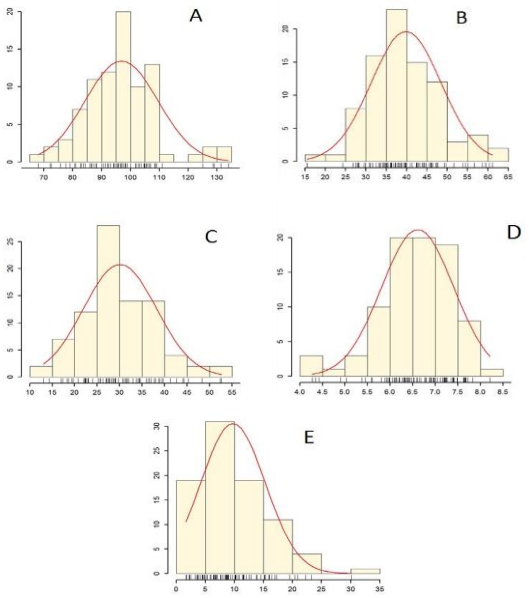

Fig 1. Phenotypic distribution of five different traits under field condition, (A) plant height (cm), (B) raceme height (cm), (C) number of pods on main raceme, (D) pod length (cm), and (E) flower and pod abortion.

Table 2. Significant markers at 0.01 percentile associated with five different agronomic traits under natural heat stress condition.

Traits/Markers	Chrom-osome	Position	P	R^2 (%)	Allele 1			Allele 2			Heterozygous Allele		
					All-eles	# Obs	Mean	All-eles	# Obs	Mean	All-eles	# Obs	Mean
Plant height													
chrC08_32368215	C08	32368215	2.40E-05	0.24	A	1	134	G	77	96.8	R	7	92.44
chrC03_545192	C03	545192	2.61E-05	0.24	G	52	94.1	T	18	103.58	T	18	103.58
chrCnn_rand_78509836	Cnn-rand	78509836	2.99E-05	0.23	C	7	98.4	T	72	95.1	Y	6	116.51
Raceme height													
chrC01_15689071	C01	15689071	1.74E-05	0.22	G	59	39.7	T	20	36.86	T	20	36.86
chrC01_15689086	C01	15689086	5.77E-05	0.2	C	55	40	T	25	37.5	Y	5	50.11
chrA02_1133295	A02	1133295	8.39E-05	0.2	A	25	37.5	T	55	40	W	5	50.11
chrC01_26101660	C01	26101660	1.18E-04	0.19	A	25	37.5	T	55	40	W	5	50.11
Pods on main raceme													
chrA10_rand_2092893	A10_rand	2092893	9.42E-05	0.21	A	43	27.9	G	28	30	R	14	37
chrA10_rand_2092900	A10_rand	2092900	9.42E-05	0.21	C	62	30.7	T	12	33.1	Y	11	23.06
chrA09_26370461	A09	26370461	1.27E-04	0.21	A	71	29	T	2	27	W	12	37.17
chrAnn_rand_10002128	Ann-rand	10002128	2.98E-04	0.19	C	2	27	G	71	29	S	12	37.17
chrAnn_rand_10002131	Ann-rand	10002131	2.98E-04	0.19	A	11	32.5	G	64	30.8	R	10	22.63
Pod length													
chrC02_33478452	C02	33478452	7.34E-06	0.26	A	26	6.4	G	47	6.9	R	12	5.87
chrC09_43471822	C09	43471822	1.24E-05	0.25	A	25	6.5	G	44	6.9	R	16	5.97
chrAnn_rand_11544915	Ann-rand	11544915	3.50E-05	0.23	A	38	6.6	G	39	6.8	R	8	5.62
chrC03_58651519	C03	58651519	3.72E-05	0.23	A	11	7	G	68	6.7	R	6	5.37
Sterile/aborted pod													
chrA03_4072206	A03	4072206	5.20E-06	0.27	A	9	14.9	T	40	10.1	W	36	8.06
chrC02_13281695	C02	13281695	9.16E-06	0.26	A	16	7.7	G	20	13.5	R	49	8.9
chrC02_13209276	C02	13209276	2.22E-05	0.23	A	70	8.9	C	4	9	M	11	15.67
chrC02_13209244	C02	13209244	2.22E-05	0.23	C	4	9	T	70	8.9	Y	11	15.67
chrA10_1216770	A10	1216770	1.19E-04	0.16	C	67	39.9	G	3	29.8	S	15	41.9

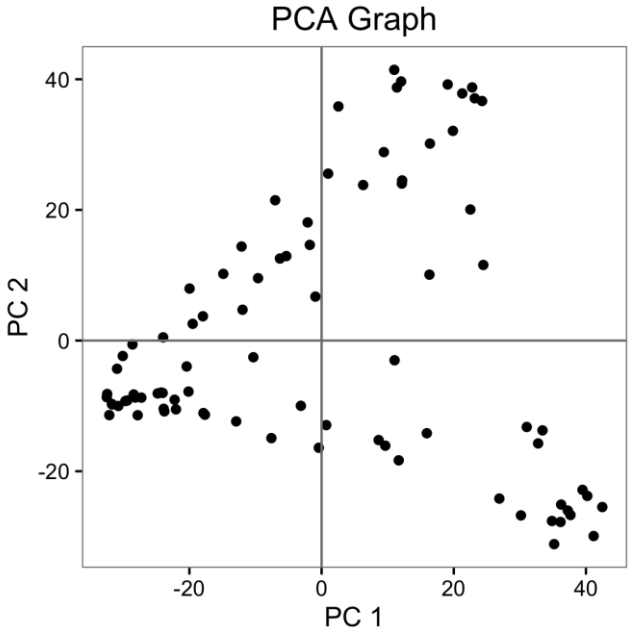

Fig 2. PC graph of the first two principal components using 37,269 polymorphic SNPs. The X-axis is representing PC1 and Y-axis is PC2. This graph explains the similarities among the germplasm accessions and the overall population structure.

Table 3. 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	52.2
			C03	0.54	
			C06	5.17	
			C07	38.5	
			C07	6.80	
			C08	32.3	
Main raceme height	36	11	A02	1.13	71.8
			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 on main raceme	25	7	A09	26.3	53.2
			C01	3.05	
			C01	9.23	
			C03	8.00	
			C09	3.59	
			A10_ rand	2.09	
			Ann_ rand	10.0	
Pod length	38	11	A03	4.12	73.5
			A05	20.3	
			A09	32.4	
			A10	16.4	
			C01	14.8	
			C01	16.9	
			C03	1.38	
			C03	12.3	
			C07	40.1	
			C09	43.4	
			C02_ rand	3.64	
Sterile/aborted pod	35	7	A05	22.8	61.0
			A07	1.11	
			C02	13.2	
			C04	5.45	
			C04	5.46	
			C05	22.9	
			C04_rand	0.98	

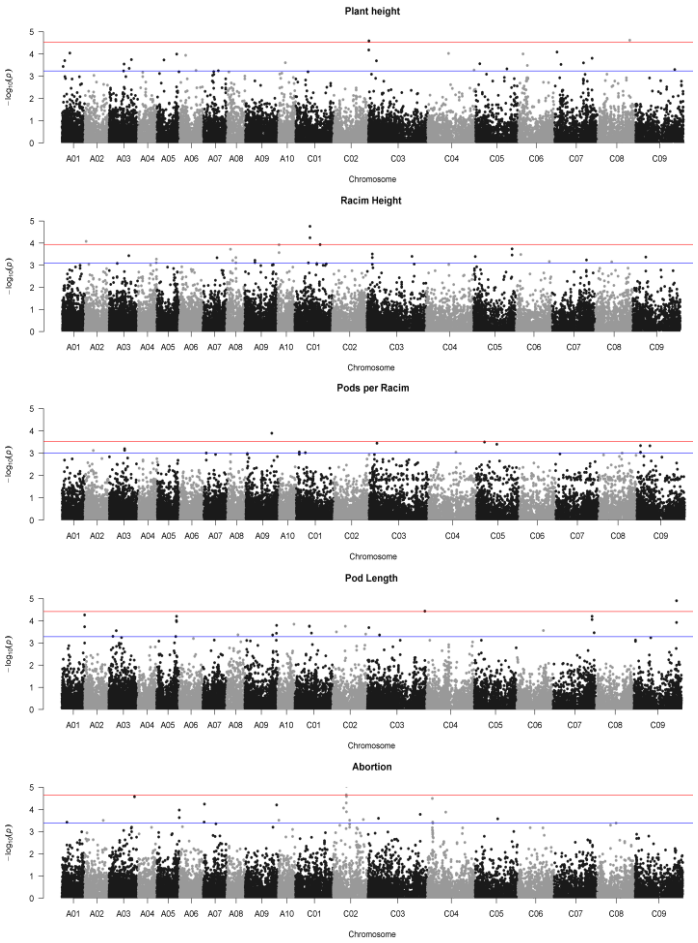

Fig 3. Manhattan plots showing p values across 19 chromosomes of *B. napus* for SNP markers associated with five different traits.

B-deficiency and pod development were present in the QTL region (Supplementary table S4).

Variation in sterile/aborted pod was associated with four significant markers at 0.01 percentile ($p \leq$ 5.20E-06, Table 2, Fig. 3). These markers were located on chromosome A03 (4.07 Mbp) and C02 (13.20 Mbp). Further 31 markers were identified with significance at 0.1 percentile tail of the empirical distribution ($p \leq$ 2.57E-05, Supplementary table S3). A stepwise regression was performed, and 7 QTL regions were identified that explained 61.0% of phenotypic variation of sterile/aborted pod (Table 3, Fig. 4). Many candidate genes known to be involved in organ abortion were also identified. These genes included heat shock proteins, genes associated with male sterility, embryo abortion, pollen abortion, and reduced flowering fertility (Supplementary table S4).

Discussion

Rapeseed/canola is a cool season crop and is sensitive to heat stress (Morrison, 1993). Increasing temperatures and heat stress are a growing concern for canola production. Therefore improvement of the crop against heat stress traits may help the adaptation and expansion of the geographical range of cultivation of this crop. To achieve this, a genome-wide association study was conducted to identify significant markers closely associated with heat stress effected traits, that can be helpful for marker assisted selection. The germplasm accessions flowered within 40-60 days of planting were considered as spring type. These accessions were exposed to natural heat stress in the field during the reproductive stage. Many studies on heat stress under controlled conditions are available, however very limited studies on heat stress affected traits of canola under field conditions are available. The germplasm used in this study are originated/obtained from 13 countries (3 continents), and have relatively higher genetic diversity. These genotypes represent the most available spring type diversity in our germplasm collection. This diversity will generate a better mapping resolution and help to identify QTL regions that can be used for marker assisted selection (MAS). These genotypes respond differently to heat stress and lead to a higher phenotypic variability.

We studied plant height, main raceme height, number of pods on main raceme, pod length, and sterile/aborted pod of canola under field conditions. The phenotypic data is from a single year field study. This is similar to Hwang et al. (2014), who conducted a genome-wide association study of seed protein and oil content in soybean with one-year field trial. 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 for association mapping in Scandinavian winter wheat for seed yield, plant height, and

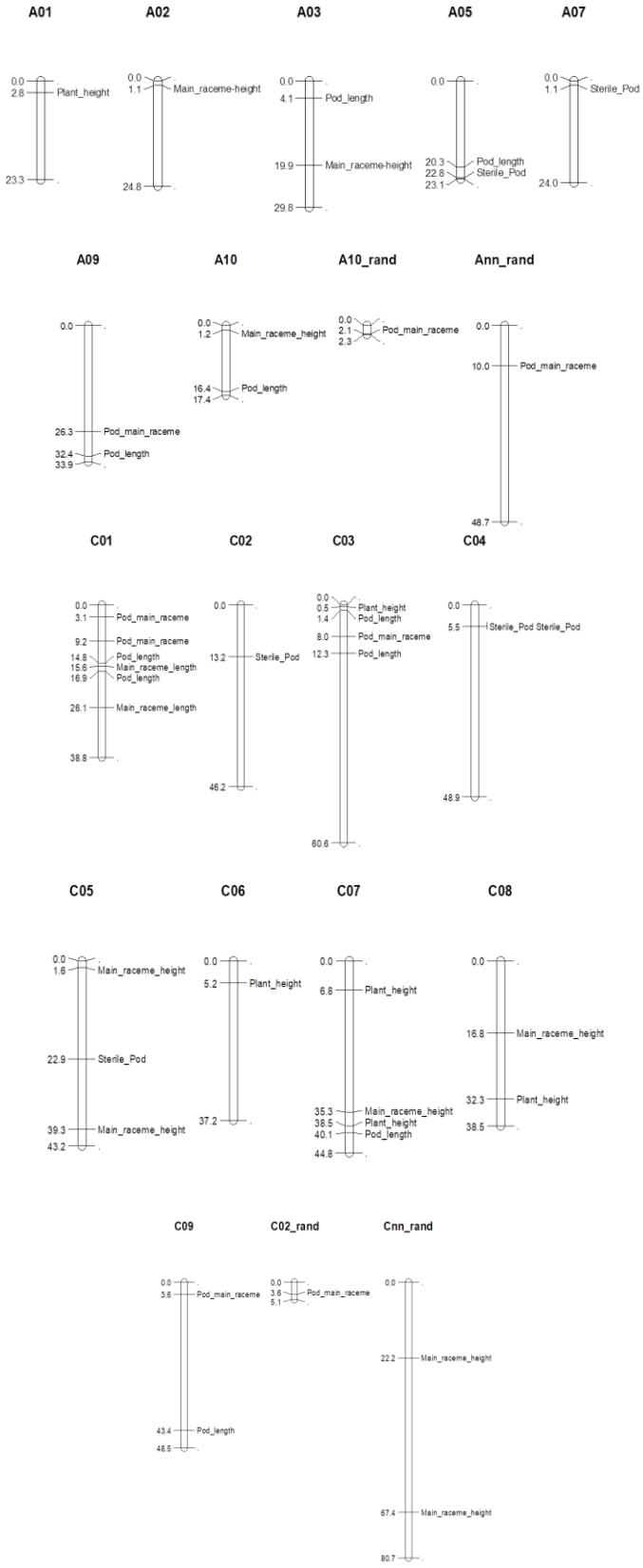

Fig 4. The QTL positions of plant height, main raceme height, pods on main raceme, pod length, and sterile/aborted pods located on different chromosomes of *B. napus.*

traits important for second-generation bioethanol production. Even though studies based on multiple years might be beneficial for QTL identification that could effectively be used for MAS. However, the drawback would be availability of heat stress during reproductive stage in multiple years. Since the intended application is minimizing the generation advancement effort, by using marker assisted selection, data from one year should suffice.

The plant height and main raceme height varied significantly among the genotypes. Heat stress negatively affects the 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 significant pod sterility and pod abortion (Morrison, 1993). Variable flower and pod abortion were also observed in our study. Variability of pod abortion due to heat stress is also reported in other crops such as 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). Heat stress affects the tapetum layer of pollen and reduces the nutrition supply, especially during microspore development. This shortage of nutrient supply affects the male gametogenesis, and hamper the formation of microspore cells and ultimately causes pod abortion (Ma et al., 2005).

Multiple genes, and biochemical and metabolic pathways govern the heat stress tolerance in plants. For example, antioxidant activity, membrane lipid unsaturation, gene expression and protein translation, stability of protein, and accumulation of compatible solutes play 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., 2000; Nuttal et al., 1992).

In this study, a genome-wide association study (GWAS) was conducted to identify significant markers associated with the five agronomic traits that are known to be affected by heat-stress. GWAS helps to identify candidate genes for each trait of interest in a population. It is also a powerful tool to identify QTL associated with various traits of crop species (Huang et al., 2012). The phenotypic variation of many complex traits is influenced by multiple QTL and association mapping helps to identify molecular markers that are closely linked to the QTL or genes controlling the traits (Li et al., 2011). We used single nucleotide polymorphism (SNP) markers for our association mapping study. SNPs are frequently used markers, which contribute the majority of genotyping in different crop species including *B. napus* (Trick et al., 2009).

About 37,000 SNPs were used in this study. The missing data of the SNPs was imputed to increase the map resolution of the study and to map the causal variant of the analysis. To protect from spurious marker-trait associations (Price et al., 2010), we tested different regression models that include structure and/or relatedness. Initially, a large number of significant markers were identified associated with heat stress traits. Further, bootstrapping identified only a few QTL significantly associated to heat stress affected traits (Mamidi et al., 2014). This is similar to earlier research, where 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%. The significant marker was selected around 100 kbp of each side of the major QTL due to the lower LD of the studied canola accessions (Monika et al., Unpublished).

Plant height is an important trait of canola affected by heat stress. Heat stress affects the photosynthesis (Crafts-Brandner and Salvucci, 2002) and produce Reactive Oxygen Species (ROS) (Hasanuzzaman et al., 2013) which severely reduces plant growth and development. Plants accumulate protein and osmolytes under heat stress, which help to continue photosynthesis by enhancing the activities of many antioxidants like superoxide dismutase (SOD), catalase (CAT) and peroxidise (POD), and by scavenging the harmful ROS (Warich et al., 2012). In our study, the combined phenotypic variation of plant height due to the major QTL was about 53%. The heavy metal transport/detoxification superfamily protein gene was found in chromosome C03 which was only 4 kbp apart from the major QTL at 545 kbp. This gene is associated with plant growth and development and helps to sustain growth under abiotic stress conditions (Hall 2002). Many other genes were found associated with heat stress such as gibberellin 2-oxidase 8 which regulates plant growth (Lo et al., 2008), ethylene-regulated nuclear protein (ERT2), which regulates plant growth and development through cell elongation and cell division (Sakai et al., 1998), ABC-2 type transporter family protein is involved in plant growth, development and response to abiotic stresses (Kang et al., 2011). Other genes associated with plant growth and development such as 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 also identified.

Raceme height is correlated with the plant height that is ultimately associated with yield of canola. GWAS revealed 36 significant SNP markers and eleven QTL on chromosomes A02, A03, A10, C01, C05, C07 and C08. Many candidate genes were identified that are associated with raceme height and are involved in different physiological process. Of these candidate genes, Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family proteins 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 et al., 2006), protein kinase family protein involved in stem elongation and vascular development (Matschi et al., 2013), auxin response factor 1 regulates plant growth and development (Li et al., 2016), mitogen-activated protein kinase acts 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 and Van Der Straeten, 2014).

Number of pods on main raceme depends on the pod development and rate of aborted pods. 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 in the pollen receptivity of the stigma (Rao et al., 1992). Many genes are involved in the variation of number of pods per plant. We have identified seven significant QTL that explained 53.2% of total phenotypic variation. One significant marker on chromosome C03 at 8.00 Mbp is located in *Brassica* gene BnaC03g15870D that contain protein kinase superfamily protein, which is involved in pollen abortion of crops (Radchuk et al., 2006). Many other candidate genes were identified that are associated with the variation of number of pods per plants. Among the candidate genes, basic helix-loop-helix (bHLH) DNA-binding superfamily protein that is

involved in the development and dehiscence of seed and pod (Hudson and Hudson, 2015), protein kinase superfamily protein is involved in pollen abortion (Radchuk et al., 2006), pyruvate kinase family protein 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 and Pandey, 2016), chaperone DNAJ-domain superfamily protein is involved in male sterility (Yang et al., 2009), DNAJ heat shock N-terminal domain-containing protein that increases tolerance to heat and prevents fruit drop (Zhao et al., 2015), proline-rich family protein associated with flower and pod development (Giorno et al., 2013), adenine nucleotide alpha hydrolases-like superfamily protein is involved in male sterility (Mok and Mok, 2001), homeodomain-like protein regulates 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 found associated with early embryo abortion (Zhang et al., 2014).

Pod length is one of the indicators of seed yield in *Brassica*. Pod length is also affected by heat stress. High temperature reduces the photosynthetic capacity (Crafts-Brandner and Salvucci, 2002) and increase pollen abortion (Zhang et al., 2014), which in turn affects the growth and development of pod. We have identified 11 QTL associated with pod length in relation to heat stress. The QTL together explained a phenotypic variation of 73.5%. One 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 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 and Hong, 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 and Nayyar, 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.

Sterile/aborted pod is significantly affected by heat stress, and causes significant yield loss of *Brassica*. Thirty-five SNPs were identified associated with sterile/aborted pod on different chromosomes. Stepwise regression identified seven significant QTL located on chromosome A05, A07, C02, C04, and C05. The markers chrC04_5456736, and ChrC04_rand_988002 were 4kb 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 genes associated with these QTLs are F-box family proteins 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 proteins are involved in male sterility and ultimately cause pod abortion (Mok and Mok, 2001).

Material and methods

Phenotyping

A total of 84 spring type *B. napus* accessions were used in this study (Supplementary table S1). The accessions were obtained from Germplasm Resources Information Network (GRIN) (http://www.ars-grin.gov/npgs/searchgrin.html), and were grown in the field at Prosper, North Dakota during summer 2014. The experiment was laid out in a randomized complete block design (RCBD) with 3 replications. Three plants per replication were tagged randomly during flowering time for data collection. During the pod initiation time (1st week to 3rd week of July) the air temperature was about 35ºC (https://ndawn.ndsu.nodak.edu), which created a natural heat stress for about 20 days (Supplementary table S2). Data on plant height (cm), raceme height (cm), number of pods on the raceme, pod length (cm), and sterile/aborted pod were recorded at the physiological maturity stage of the crop.

Genotyping and association mapping (AM)

Genomic DNA was extracted from a collection of 366 individuals representing the entire canola diversity available at North Dakota State University (Monika et al. Unpublished) were sequenced using a Genotype-By-Sequencing protocol (Elshire et al. 2011). Briefly, the samples were digested with *ApkI* enzyme. Illumina GAII sequencer was used to sequence the sample as 100 bp single end reads from size selection of 300–700 bp fragments. Sequence alignments were performed using BWA-mem (Li et al., 2013) and SNP calling using VarScan (Liu et al., 2013). The SNPs obtained at this stage were used for further analysis. FastPHASE (Scheet and Stephens, 2006) was used to estimate the missing alleles. The marker data for these 84 spring type individuals was further cleaned for minor allele frequency of 5%, below which markers were removed. Finally, 37,269 SNPs were subsequently used for this analysis.

Structure analysis, kinship, and model testing

Population structure was controlled using principal components (PC) that were 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 as the proportion of shared alleles for all pairwise comparisons within the population (Zhao et al., 2007). 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. All the analyses were performed in TASSEL. 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). Significant markers were identified based on the p-value of a marker within 0.01 and 0.1 percentile tail of 10,000 bootstraps (Mamidi et al., 2014; Gurung et al., 2014; Kertho et al., 2015). Significant markers were selected from the selected best models, and

Mahhattan plots were constructed using $-\log_{10}$ of *P*-values against chromosome location using qqman package in R (Turner 2014).

Identification of QTL and candidate genes

Stepwise regression was implemented in SAS using SAS REG procedure to estimate the combined variation (r^2) explained by all markers and to select the minimum number of markers that can be used for marker assisted selection, and that define a 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.

Conclusion

Rapeseed/canola is a heat sensitive crop and can cause significant yield losses at high temperatures. Additionally, the cultivation in U.S. is limited to North Dakota. For improving the crop productivity under heat stress and increasing the cultivation area, there is an urgent need to develop genotypes that are resistant to heat stress. For this, we have included all available spring type germplasm and evaluated in field conditions, where heat stress was observed during the flowering stage. As anticipated, we identified multiple QTL for each of the five agronomic traits. The markers can be used for MAS, while candidate genes within vicinity of QTL can be used for additional functional studies.

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