Omics Approaches to Decipher Nitrogen Response in Bread Wheat

By

TAYYABA ANDLEEB

Registration No. 03041613005

Department of Plant Sciences,

Faculty of Biological Sciences,

Quaid-i-Azam University,

Islamabad, Pakistan

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TAYYABA ANDLEEB

Registration No. 03041613005

Department of Plant Sciences,
Faculty of Biological Sciences,
Quaid-i-Azam University,
Islamabad, Pakistan
2023

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This is to certify that the research work presented in this thesis, entitled as **"Omics Approaches to Decipher Nitrogen Response in Bread Wheat"** was conducted by **Ms. Tayyaba Andleeb** (Registration No. 03041613005) under the supervision of **Dr. Umar Masood Quraishi**, Associate Professor, Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan. The thesis has not been submitted partially/completely anywhere else for any other degree. This thesis is submitted to Department of Plant Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan in the partial fulfilment of the requirements for the degree of Doctor of Philosophy in the field of **Plant Sciences**.

Tayyaba Andleeb (Ph.D. Scholar) Signature: _____

Examination Committee

External Examiner 1

Prof. Dr. Fayyaz-ul-Hassan
Pro-Vice Chancellor
PMAS, Arid Agriculture University, Rawalpindi

Signature: _____

External Examiner 2

Dr. Malik Ashiq Rabbani
Chief Scientific Officer
Pakistan Agricultural Research Council

Signature: _____

Supervisor

Dr. Umar Masood Quraishi
Associate Professor
Department of Plant Sciences
Quaid-i-Azam University, Islamabad

Signature: _____

Chairman

Prof. Dr. Mushtaq Ahmad
Chairman
Department of Plant Sciences
Quaid-i-Azam University, Islamabad

Signature: _____

Dated: **February 22, 2023**

FOREIGN EXAMINERS

1. **Professor Dr. Jerry Roberts**
   Emeritus Professor
   University of Plymouth
   Office of the Vice-Chancellor
   18 Portland Villas | Plymouth | PL4 8AA
   Tel: +44 1752 582016
   Email: jerry.roberts@plymouth.ac.uk

2. **Professor Dr. Takashi Watanabe**
   Director, Department of Medicinal Plants
   Kumamoto University
   5-1 Oe-Honmachi, Chuo_ku, Kumamoto, 862-0973,Japan
   Tel: +81-96-371-4781, Fax: +81-96-371-478
   Email: wtakashi@kumamoto-u.ac.jp

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Supervisor:

Dr. Umar Masood Quraishi
Associate Professor
Department of Plant Sciences
Quaid-i-Azam University
Islamabad

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Knowledge is the root of all good.

Dedicated to

my father

Muhammad Nawaz

and my mother

Ruqia Begum

for instilling the importance of hard work and higher education

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LIST OF ABBREVIATIONS

N	Nitrogen
NUE	Nitrogen use efficiency
AM	Association mapping
GLM	General linear model
MLM	Mixed linear model
FarmCPU	Fixed and random model circulating probability unification
GWAS	genome-wide association study
rMVP	Memory-efficient Visualization-enhanced Parallel-accelerated
QTL	Quantitative trait loci
SNP	Single nucleotide polymorphism
LD	Linkage disequilibrium
MTAs	Marker trait associations
TASSEL	Trait Analysis by aSSociation Evolution and Linkage
DNA	Deoxyribonucleic acid
IWGSC	International Wheat Genome Sequencing Consortium
BLUPs	Best linear unbiased predictions
CIMMYT	International Maize and Wheat Improvement Center
CHL	Chlorophyll content
SPAD	Soil plant analysis development
RSI	Relative SPAD index
NDVI	Normalized difference vegetative index
RNDVI	Relative normalized difference vegetative index
CT	Canopy temperature
CTD	Canopy temperature depression
PH	Plant height
TN	Tiller number
TP	Tiller per plant
FLA	Flag leaf area
SL	Spike length
GpS	Grains per spike
TKW	Thousand kernel weight
GY	Grain yield
BM	Biomass
HI	Harvest Index
NAE	Nitrogen agronomic efficiency
g	Grams
cm	Centimeter
RNA	Ribonucleic acid
cDNA	Complementary DNA
dNTPs	Deoxyribonucleotide triphosphates

LIST OF ABBREVIATIONS

HN	High nitrogen
LN	Low nitrogen
DE	Direct effect
IE	Indirect effect
TE	Total effect
MLR	Multiple linear regression
GYC	Grain yield components
RT	Root traits
RL	Root length
RSA	Root surface area
RMN	Root mean number
ha	Hectare
min	Minute
sec	Seconds
WT	Wild type
RNAi	Ribonucleic acid interference
NAM	No apical meristem
NAM RNAi	*NAM* transgenic line
DAA	Days after anthesis
GPC	Grain protein content
DE	Differentially expressed
DEGs	Differentially expressed genes
TF	Transcription factor
GO	Gene ontology
GS	Glutamine synthetase
GOGAT	Glutamine-2-oxoglutarate aminotransferase
ASN	Asparagine synthetase
GDH	Glutamate dehydrogenase
ASN	Asparagine
GLN	Glutamine
GLU	Glutamate
AMT	Ammonium transporters
AAT	Amino acid transporters

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Omics Approaches to Decipher Nitrogen Response in Bread Wheat

Abstract

Nitrogen (N) plays significant role to improve above ground biomass, grain yield, grain production and grain protein content. It is used for synthesis of amino acids, signaling molecules and storage molecules as well as being essential for number of metabolic processes. The synthetic nitrogen fertilizer improves crop performance and yield related traits but most of crops absorb merely 30–50% of applied N fertilizer, depending on the environment, plant genotype and soil type. More than 50% of applied N fertilizer is not utilized by crops and lost into environment ultimately leading to ecosystems' destabilization. Even in intensive farming systems, total crop production has not been improved according to chemical fertilizer application rate and leads to low NUE and environmental pollution. These facts highlight the potentials and challenges of improving global food security while implementing novel strategies not only to improve crop yield but also to reduce N inputs is concurrent in future. The nitrogen uptake, utilization and remobilization in wheat needs to be further explored at agro-physiological, biochemical, and molecular levels for introgression in future breeding programs. Our first study aimed to unravel the genetic composition of nitrogen response in a diverse germplasm consisting of landraces, green revolution, post green revolution, elite cultivars, and CIMMYT advance cultivars using 90K SNP array by employing general linear model, mixed linear model, and fixed and random model circulating probability unification based genome-wide association mapping. Seventy two significant marker trait associations were selected for gene identification conferring chlorophyll content, normalized difference vegetation index, flag leaf area, plant height, tiller number, grain yield, biomass, harvest index, grains per spike and nitrogen agronomic efficiency. Genes corresponding to the significant MTAs were retrieved as candidate genes, including members of the transcription factor families and protein kinases.

The second study aimed to identify the major grain yield components and root traits and their level of contribution for yield maximization under variable N supplies through multiple linear regression and building their path model using LISREL software. It computes multiple linear regression (MLR) to show the interaction between independent (grain yield components and root traits) and dependent (grain yield) variables in the form of direct effect

(DE), indirect effect (IE) and total effect (TE). The tiller number, days to maturity, nitrogen use efficiency and root length showed high correlations and direct effects on GY under variable N application. Multiple linear regression (MLR) analysis by building path model is an effective way to predict improvement in grain yield as it showed the intensity of association between two or more yield related traits and indicated relative importance of each trait.

The third study aimed to demonstrate the impact of nitrogen use efficiency to mitigate terminal heat stress in bread wheat under variable nitrogen applications. Nitrogen (N) deficiency and heat stress (HS) are major abiotic stresses that affect the quantity and quality of wheat grains. Twelve wheat varieties were evaluated in 2016–2017 and 2017–2018 at the National Agricultural Research Centre (NARC), Islamabad, Pakistan. The experiment was divided into three sets, i.e., N120 (120 kg N/ha), N60 (60 kg N/ha) and N0 (0 kg N/ha), based on the nitrogen fertilizer application. The strong positive correlation of RSI and RNDVI with grain yield at $R^2 = 0.73$ and $R^2 = 0.49$ suggest that these parameters can be used as efficient and precise selection criteria for identifying nitrogen-use-efficient wheat varieties under terminal heat-stress conditions. This work will help the researchers to identify and develop nitrogen-use efficient and thermos-tolerant wheat cultivars by minimizing the negative impacts of heat stress at the anthesis stage.

The fourth study aimed to demonstrate how related *NAM* genes control nitrogen remobilization at the molecular level in bread wheat. We carried out a comparative transcriptomic study at seven time points (3, 7, 10, 13, 15, 19 and 26 days after anthesis) in wild type and *NAM* RNA interference (RNAi) lines with reduced *NAM* gene expression. Approximately 2.5 times more genes were differentially expressed in WT than *NAM* RNAi during this early senescence time course (6,508 vs 2,605 genes). In both genotypes, differentially expressed genes were enriched for GO terms related to photosynthesis, hormones, amino acid transport and nitrogen metabolism. However, nitrogen metabolism genes including *glutamine synthetase* (*GS1* and *GS2*), *glutamate decarboxylase* (*GAD*), *glutamate dehydrogenase* (*GDH*) and *asparagine synthetase* (*ASN1*) showed stronger or earlier differential expression in WT than in *NAM* RNAi plants, consistent with higher nitrogen remobilisation. The current thesis reports fundamental knowledge of molecular basis of nitrogen response in bread wheat.

Chapter #1

Introduction and Review of Literature

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1.1. Socio-economic importance of wheat

The advent of agriculture has contributed to the progression of human civilization from the prehistoric to the modern eras. Agriculture feeds around 7.8 billion people on planet Earth. Continuous selection of desirable agronomic traits have resulted in increased yield, allowing us to withstand food shortages (Eckardt, 2010). There has been a lot of discussion about the effects of expected increase in world population from 7.4 billion in 2017 to 9.7 billion in 2050 on global food demand (Fukase & Martin, 2020). The global food security is largely dependent on global cereal production. Cereals contributes approximately 20-30% of total dietary calories (Alexandratos & Bruinsma, 2012). Cereals are the domesticated members of family *Poaceae* including rice, maize, wheat, barely, oat, sorghum, millet and rye. Followed by rice and maize, wheat is the most important food crop (Green et al., 2012; Hernandez et al., 2012; Peleg et al., 2011). Among cereals, wheat has a significant role in ensuring global food and nutrition security as it contributes a fifth of the world's food calories and protein (Grote et al., 2021). Wheat is the most cultivated crop across the world on an area around 217 million hectors annually (Erenstein et al., 2022). Compared to other cereals, it is one of the largest internationally traded crops (Atchison et al., 2010). The global wheat production was, on average, around 778 million tons in year 2020–2021 (FAO, 2022) as shown in Figure 1.1.

In the developing regions of the world, food demand is growing 1% each year. It varies from 27kg in East and South Africa to 170kg in China and Central Asia which contribute 50% of the total food production and 53% of the total harvested area (Shiferaw et al., 2013b). Wheat is reported to be cultivated around 10,000 years ago as part of Neolithic Revolution which was a transition from the nomadic to an agrarian lifestyle (Dubcovsky & Dvorak, 2007; Faris, 2014; Lev-Yadun et al., 2000). Wheat major producers are Europe and North America in the developed world and Asia in the developing world (Grote et al., 2021). Major exporters of wheat are Australia, Argentina, Canada, Europe, Kazakhstan, Russia, Ukraine and the United States (http://www.fao.org/faostat). The world's area used for growing wheat has fluctuated between 200 and 240 million hectares since 1961. Wheat production reached its high around 1980 and has been oscillated downward to the current 217 M ha level after 1980. The growth in worldwide wheat production is explained by steady gains in wheat yield given the relatively

stable wheat acreage including a slight drop over the last 50 years. From the early 1960s, global average yield of just over 1 ton/ha to the current 3.5 tons/ha. Yields have gradually improved, almost tripling global wheat production during that time (Erenstein et al., 2022).

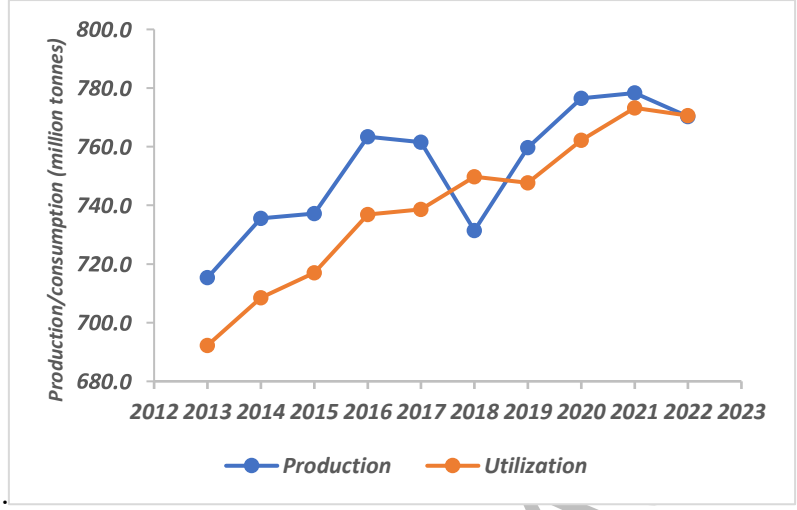

Figure 1.1. Dynamics of world wheat production and utilization from 2013 to 2022 in million tonnes (Source: http://www.fao.org/faostat).

Wheat production is vital to Pakistan economy being the major staple crop of the Pakistani nation (Sher & Ahmad, 2008). It contributes around 1.8 % to GDP and accounts for 9.2 % of the value added to agriculture. In Pakistan, 40% (9 million hectares) of the total arable area is used for wheat cultivation. In term of acreage, wheat is the largest grain crop of Pakistan contributing 75% of total grain production (Farooq et al., 2000). Pakistan is ranked 8th in term of wheat export. A record-breaking production of wheat was obtained (~27.293 million tonnes) in year 2020-21 with an increase of 8.1 percent above the previous year's production (Figure 1.2).

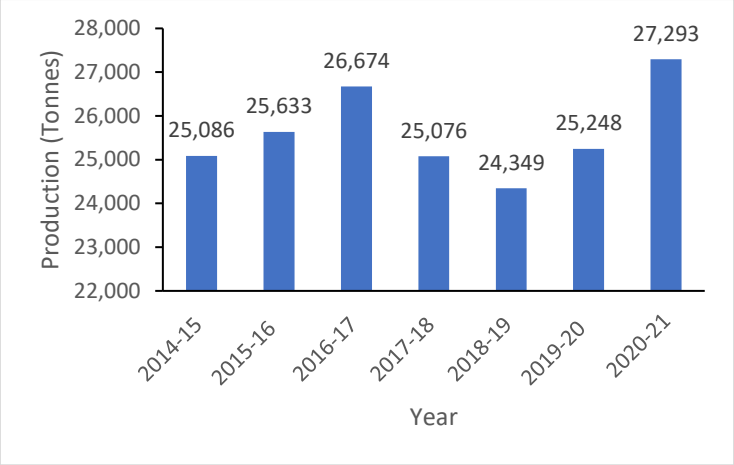

Figure 1.2. Dynamics of wheat production in Pakistan in last seven years (2015 to 2021) in tonnes (source: Pakistan Bureau of Statistics and government of Pakistan 2020-21).

1.2. Wheat genome and evolutionary history

Wheat is an allopolyploid with three sub-genomes A, B, D while each sub-genome has seven chromosomes making n=21 (Feldman, 2000; Kimber et al., 1987). Wheat genome is about 17000 Mb which is quite large with high (~80%) proportion of repetitive sequences (Gupta et al., 2008). Deletion mapping has demonstrated that the wheat genome has rich genetic regions (Gill et al., 1996). Inter and intraspecific hybridization and polyploidization are responsible for evolution of genus Triticum (Gustafson et al., 2009). The wild progenitors of enkiron wheat and emmer wheat are found together in core area of Fertile Crescent (Lev-Yadun et al., 2000). It has two main types, hexaploid *Triticum aestivum L.* (Bread Wheat) and *Triticum durum* (Durum Wheat), which contributes approximately 95% and 5% of total world wheat production respectively (Padulosi, 1996; Peng et al., 2011).

Triticum aestivum L. is derived from crossing between a diploid wheat *Aegilops tauschii* and *Triticum turgidum* ssp. *Dicoccoides* a tetraploid wild emmer, which makes it an allohexaploid crop (Dubcovsky & Dvorak, 2007; Foulkes et al., 2009b; Matsuoka & physiology, 2011). High Structural conservation and sequence similarity was observed in wheat relatives by comparative gene analysis (Hernandez et al., 2012). The sub-genomes of hexaploid bread wheat and extant diploid and tetraploid wheat relatives showed dynamic gene loss, gain and duplication across the genomes since the divergence of wheat lineages (Figure 1.3).

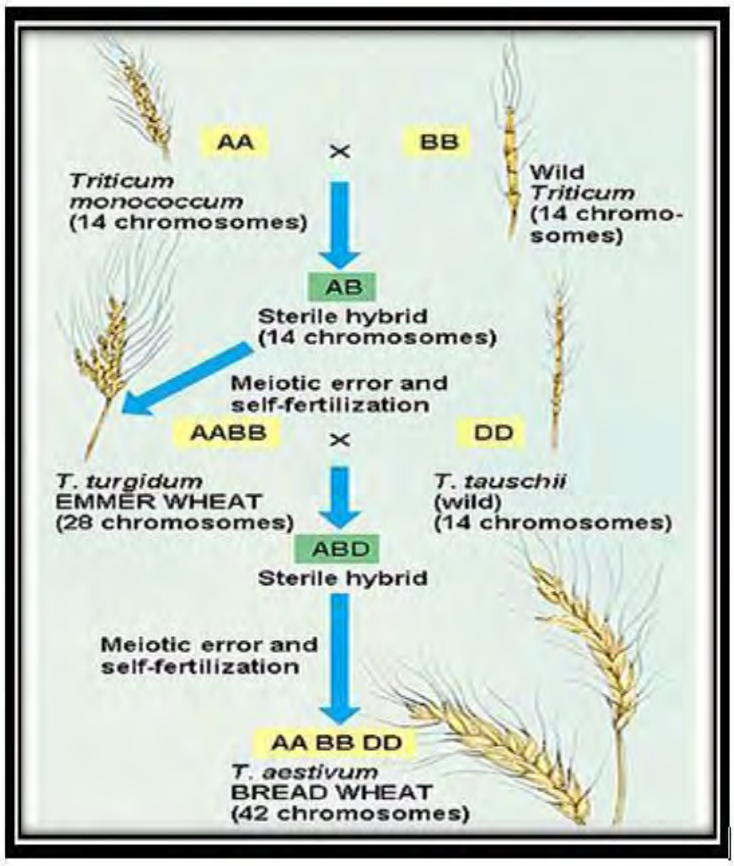

Figure 1.3. The wheat evolution from prehistoric grasses to modern macaroni and bread wheat source:(Schemske, 2000).

1.3. Wheat growth stages

Hanft and Wych (1982) has divided wheat growth into four stages, i.e., growth stage E (from germination to emergence), growth stage 1 (tillering and stem elongation), growth stage 2 (from stem elongation and booting to heading), and last growth stage 3 (from anthesis to grain filling and eventually physiological maturity). These stages duration depends upon environmental conditions as well as genotype.

Wheat lifecycle starts by seed imbibition and after that radicle and coleoptile emerges with seed sprouting. Radicle develops into seminal roots and coleoptile elongates, forming first leaf which shows the start of seedling stage. After seedling stage, tillers start to emerge from auxiliary buds and thus growth stage 1 is started. Tiller formation is considered very important phase of wheat development as it determines crop yield. Tillering stage lasts usually for 10-20 days after the emergence stage. Tillering stage is considered ended after the production of new leaves stop to curb further tillering formation. By the start of growth phase 2, i.e., reproductive stage, sexual organs start to develop. Each tiller elongates its internode to form the stem. When

a small head start developing inside flag leaf sheath, booting stage is initiated. After 10-20 days of booting, heading stage starts with emergence of head from the flag leaf. After 2-5 days of heading stage, anthesis is initiated. As we know that wheat is usually self-fertilized, so after initial cellular division, amyloplasts and endosperm cells are formed. Initial phase after fertilization is lag phase, after which, grain filling lasts for 20 to 30 days. Grain filling first step is water riper or milky phase in which, starch and protein storage occur along with development of endosperm. After that, dough is developed and starch deposition in endosperm along with linear grain growth occur. In grain development stage, most of the grain weight is gained, all the proteins that are stored amid vegetative stage are then translocated to the grains. The seed dough then loses water and eventually gets hardened which provides final weight of seeds. This is ripening phase and this phase directly affects crop yield (Jones et al., 1985).

Table 1.1. General life cycle of spring wheat in Pakistan.

Development stage	Months	Days
Emergence	November, 1 to 15	0
Three leaf stage	December, 1 to 7	20
Terminal spikelet	December, 25 to 30	45
First node	January, 1 to 15	60
Booting	February, 15 to 28	90
Heading	March, 1 to 15	100
Anthesis	March, 15 to 30	100
Physiological maturity	April, 15 to May, 5	140

1.4. Wheat yield and related traits

Breeders have been trying to increase the grain yield/unit area, since the inception of agriculture. Crop yield is still the target of breeders in modern era. It's a quantitative trait that is influenced significantly by environmental factors. Yield is overall affected by a combination of physiological, morphological, genetic traits, and anatomical characters. These traits should be dissected and understood for the improvement of crop yield (Gupta et al., 2008). Following equation is used to check the extent of grain yield divergence.

$$GY(\text{unit area}) = \text{No. of plants(per unit area)} * \text{Tiller Number} * \text{Sp. S} * \text{KW}$$

Where; ***GY****= grain yield;* ***Sp.S****=spikelet per spike;* ***KW****= kernel weight.*

Different grain yield components are affected eventually by each growth phase of plant (Figure 1.4).

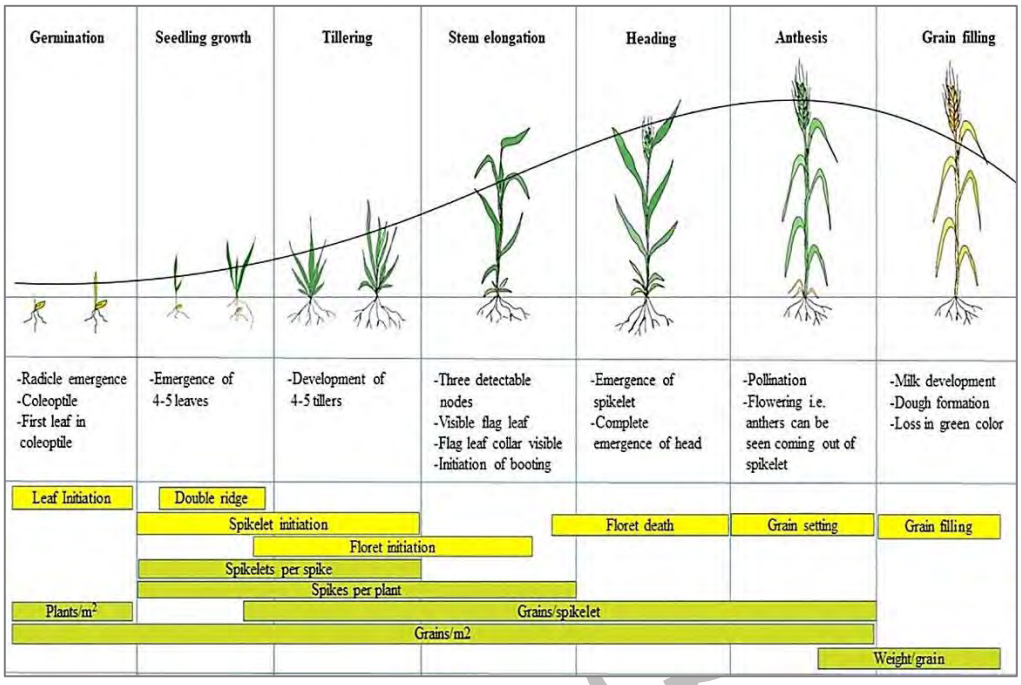

Figure 1.4. Wheat development stages along with the associated yield components at each developmental stage (Khadka et al., 2020).

1.5. Factors affecting wheat production

Wheat is one of the fewest field crops that is planted across a large range of agro-climatic conditions. This range of variations leads to many types of abiotic and biotic stresses which affect wheat growth, development and yield. Climate change and global warming have recently had a significant negative impact on the productivity of agricultural crops worldwide cultivated in tropical and subtropical regions due to the emergence of numerous new biotic and abiotic stressors. The wide range mechanisms have been adopted by wheat plants to counter different types of abiotic and biotic stresses. The biotic factors include non-parasitic and parasitic diseases such as weeds, pest, bacteria, fungi and algae that influence wheat yield to a greater extent (Figure 1.5). Seed borne diseases result in shrivelled kernels that lead to reduction of crop yield. There is also a range of viruses and pathogenic fungi that cause various root and leaf diseases in wheat (Afzal et al., 2015).

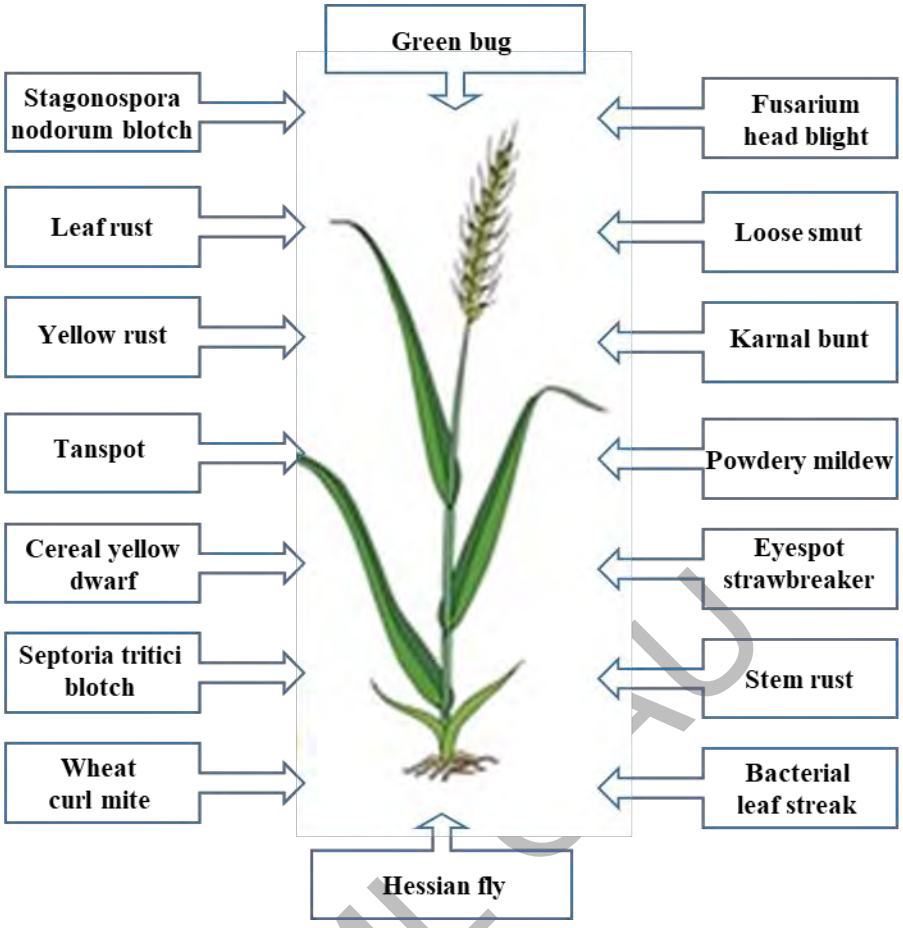

Figure 1.5. Biotic factors and causal agents that influences wheat yield.

Abiotic factors that affect crop yield includes resources (water, light, carbon dioxide, nitrogen (N), phosphorus (P), and potassium (K), stressor (salinity, soil pH, temperature and flood), and xenobiotic factors (air pollutants, organic and inorganic toxins) (Figure 1.6). The most prevalent abiotic stresses include water shortage, high temperatures, high light intensity, metal toxicity, salinity stress and nutrient deficiency. Water shortage is a serious constraint due to erratic rainfall patterns and water shortages which affect about 25% of all agricultural land. More than any other abiotic element, drought stress reduces crop productivity because it affects plant growth and development (Rad et al., 2012; Shao et al., 2009). Constant exposure to photoperiod can also reduce wheat output by shortening the grain filling duration (Abhinandan et al., 2018) Heat stress affects about 40 percent of the irrigated land used for wheat cultivation (Reynolds et al., 2001). With every 1°C increase in temperature over 15°C, wheat yield decreases by six percent (Asseng et al., 2013). Due to anthropogenic activities, almost 20% of the agricultural land has been affected. The rapid industrialization has resulted in an increase in xenobiotics including air pollutants, organic, and inorganic toxins, which negatively

influence quality and quantity of grain harvest. Grain yield improvement under reduced land and water resources and adverse environmental conditions is a daunting task.

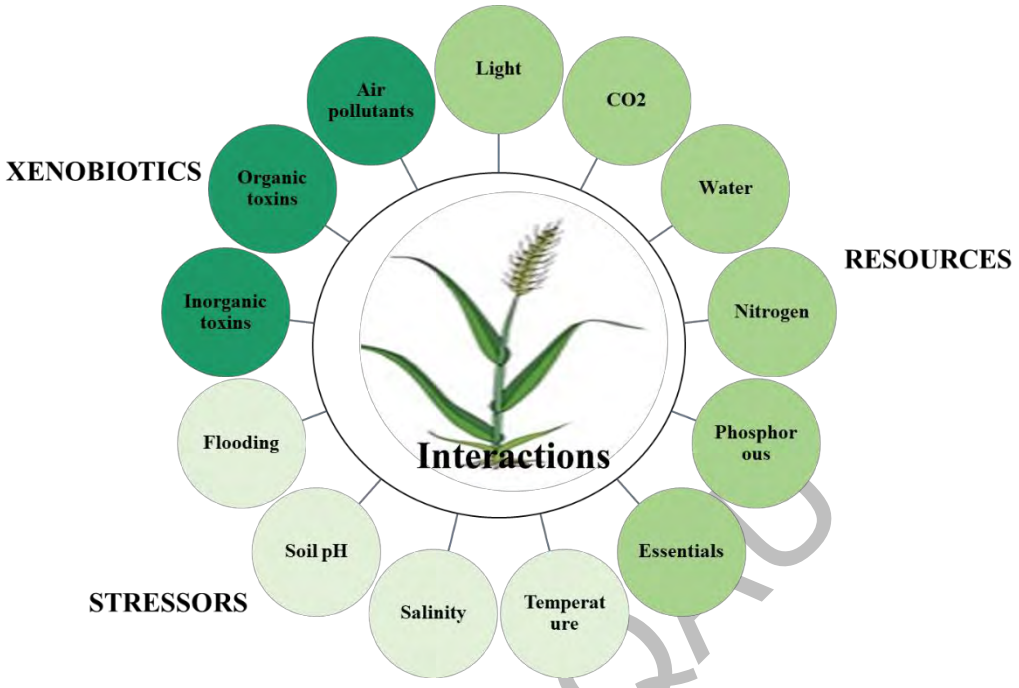

Figure 1.6. Abiotic factors influencing growth and development of wheat, source: (Willey, 2018).

1.6. Nutrient deficiency in crop plants

The deficiency of nutrients can pose a serious threat to plant production whereas its excess is also termed harmful. To understand the complex phenomenon of nutrient stress, the combined efforts of ecologists, biochemists, soil scientists, agronomists, molecular biologists, and physiologists are required. Main reasons for nutrient stress can either be low element availability or excessive concentration of elements. Sometimes, deficiency of certain elements is also caused by the excess of other elements. Various elements like nitrogen, potassium, phosphorous (Macro-elements), zinc, boron, iron, copper, molybdenum, and manganese (Micro-elements) are considered vital for plant development and growth. To enhance the productivity of plants, these macros and micronutrients are applied at various developmental stages in the form of fertilizer (Kulcheski et al., 2015).

In wheat crop, the growth, development, and yield are also affected badly due to abiotic stresses. The deficiency of macro-nutrients reduces grain yield (direct loss), and disease resistance (indirect loss) in crops. There is a significant impact on grain yield by plant's photosynthetically active canopy, which is developed by nitrogen (N). Cereal crops also require

nitrogen to produce storage proteins, a key component of grain quality. For maximum efficiency during grain filling period, regulated remobilization of canopy nitrogen is necessary. The management of nitrogen by using suitable seeds and good agricultural practices is very important for sustainable agriculture. (Anas et al., 2020).

1.7. Importance of Nitrogen

Nitrogen is an essential part of amino acids, proteins, nucleic acid, photosynthetic pigment and enzymes. (Ohyama, 2010). Nitrogen is a vital and primary driver for crop production (Suding et al., 2005), physiological processes (Evans, 1989), growth (Ågren, 1985) and reproduction (Sinclair & Jamieson, 2006). According to Bojović & Marković (2009), chlorophyll and nitrogen content in wheat leaves exhibited a significant correlation. Increased application of nitrogen fertilizer is required for maximum crop yield in order to meet increasing food demand (Hirel et al., 2007b). Worldwide food production can be doubled by increasing seven folds of N fertilizer application which ultimately has a negative impact on non-agricultural neighbouring ecosystem (Michael Beman et al., 2005). Excessive loss of nitrogen due to high rate of N-fertilizer application is one of the major factors that causes leaching of nitrogen into ground water, production and vitalization of gases like nitric oxide thus polluting atmosphere through denitrification (Conley et al., 2009; Gruber & Galloway, 2008). In the current scenario, to have a good profit margin and to avoid pollution by nitrates, use of N fertilizer must be reduced by farmers. These objectives can be achieved through efficient farming techniques and cultivation of wheat varieties with improved nitrogen response. Wheat breeders can produce superior varieties with improved N response by having sufficient knowledge of the genetic and physiological bases of N response (Chardon et al., 2012). It is therefore timely that wheat plants must use nitrogen efficiently in order to reduce deleterious impacts of nitrogen leaching on the environment (Asplund, 2014b).

In developing countries, crop productivity is mainly limited by poor access to nitrogen fertilizer. However, a substantial increase in the use of N fertilizer positively increases crop productivity in affluent countries over recent decades (Beatty et al., 2010; Hirel et al., 2007b; Ladha et al., 2005). Therefore, to adequately manage nitrogen is necessary to achieve high crop yield.

1.8. Role of nitrogen to mitigate heat stress

Nitrogen performs a very crucial role in enabling plant's tolerance against temperature stress. Light intensity is very high at elevated temperatures, which could adversely affect the plant's growth and nutrient uptake. Nitrogen also plays a key role in metabolism of photosynthetic carbon as well as utilizing the absorbing light energy (Huang et al., 2004). Besides, the fertilization of nitrogen is also reported to alleviate the harmful effects of abiotic stresses (Waraich et al., 2011). Nitric oxide (NO) despite being a membrane-permanent and highly reactive free radical, plays a key role in many physiological processes of a plant. These roles include leaf expansion, ethylene emission, seed germination, cell senescence, programmed cell death, and stomatal closure. It also performs signal molecular mediating responses to both biotic and abiotic stresses including salinity, heat and drought stress, and UV-B radiation (Hussain et al., 2022).

Besides, NO also plays a key role in direct scavenging ROS (Reactive Oxygen Species) under low or higher temperature stress, as it also acts as an antioxidant. NO is also reported to activate oxygen scavenging active enzymes thus acting as a signal in inducing plant thermotolerance. Furthermore, Uchida et al. (2002) reported by northern blot analysis that NO is responsible for the inducing the gene expression of those genes that are responsible for encoding HSP26, i.e., Heat shock protein 26 thus protecting chloroplast from oxidative stress during heat stress conditions.

1.9. Plant nitrogen assessment

Rapid assessment of nitrogen content in leaves requires dynamic nitrogen management strategies which can indicate the changes in N demand of crop throughout the growing season. The SPAD or chlorophyll meter, leaf colour chart and other simple and inexpensive alternatives can reliably and quickly monitor comparative greenness of leaf as an indicator of N status of leaf. Undoubtedly real time nitrogen management strategies can be sorted out by these tools (Ladha et al., 2005) but cannot predict actual N requirements of crop based on photosynthetic rate or expected yield and the biomass production. Consequently, SPAD meter is recognized as a tool used for detection and monitoring of N status and deficiencies in plants by comparison of CM (chlorophyll meter) readings of fully nitrogen fertilized treatment with other N treatments (Blackmer & Schepers, 1995; Varvel et al., 1997; Vidal et al., 1999). Another alternative and effective approach to identify crop N status is use of GreenSeeker sensors which

have been reported in many published studies for detection of crops' nitrogen status (D. Arnall et al., 2006; Freeman et al., 2007b; Raun et al., 2002). Many producers have reported improvement of 15% N fertilizer utilization of cereal crops by use of an efficient GreenSeeker. On the other hand, several studies have reported that nitrogen nutrition index (NNI) has potential for estimation of photosynthesis, grain amylose, grain protein content, grain yield, nitrogen requirement, partition and use efficiency of crop (Ata-Ul-Karim, Cao, et al., 2016; Ata-Ul-Karim, Liu, et al., 2016; Ata-Ul-Karim, Liu, et al., 2017; Ata-Ul-Karim, Zhu, et al., 2017; HU et al., 2014; Zhao, 2014).

To assess, N stress from canopies of plants, optical sensors like GreenSeeker are being used in agriculture that can measure near infrared (NIR) and visible spectral response (Peñuelas et al., 1994; Raun et al., 2001). An integrated optical sensor and application device called "Green Seeker" measures crop status and variable amount of crop nitrogen needed. Through NDVI (Normalized Difference Vegetation Index), vegetative index identifies the potential yield of a crop. NDVI is Plant greenness or photosynthetic activity index, the most commonly used vegetation indices (Tucker, 1979). Following equation is used for the calculation of NDVI:

$$NDVI = (NIR_{ref} - Red_{ref}) + (NIR_{ref} - Red_{ref})$$

NDVI value is influenced by many factors including total plant cover, plant soil moisture, biomass, plant photosynthetic activity, and plant nitrogen status and plant stress. Nitrogen is suggested based on crop production potential and response to extra nitrogen. Based on that, the suitable amount of nitrogen (N) is applied at the right time and place thus not only optimizing the yield but also reducing nitrogen (N) input expense.

1.10. Nitrogen metabolic pathway in plants

Metabolic pathway of Nitrogen in plants consist of several steps, including uptake, assimilation and translocation. Likewise recycling and remobilization when a plant is aging. Principal source of N for most crop and wild species is nitrates (Jolivet, 1987; (Näsholm et al., 2009). Nitrate is taken up through specific low and high affinity transporters found in the root cell membrane (Dechorgnat et al., 2011; Miller et al., 2007). Nitrate reductase is an enzyme which reduces nitrates to nitrite (Sparacino-Watkins et al., 2014) after that nitrite is reduced to ammonia by catalysis of enzyme nitrite reductase (Sétif et al., 2009). Under specific environmental conditions, root ammonia transporters (Ludewig et al., 2007) can permit a direct

ammonia uptake from soil, paddy fields of rice or in acidic habitats of forest but not specifically in wheat (Mae & soil, 1997; Salsac et al., 1987). Ammonia is produced inside plants by a number of metabolic pathways for instance phenylpropanoid metabolism, photorespiration, amino acids catabolism and utilization of N transport compounds (Hirel et al., 2011; Valentine et al., 2018).

Ammonia is available to a crop, which is further converted into amino acids by the activity of several enzymes. The first reaction of this metabolic process is catalysed by *Glutamine Synthetase* and is considered a key route helping the assimilation of mineral nitrogen into organic molecules in combination with the other enzyme glutamate synthase (Lea & Miflin, 2011; Suzuki & Knaff, 2005). Ultimately in the nitrogen assimilation cycle, it is converted into 2-oxoglutarate, a form of carbon backbone. Glutamate and glutamine are used as donor of amino groups to all the other nitrogen containing compounds including other amino acids (Lea & Miflin, 2011; Morot-Gaudry et al., 2001; Suzuki & Knaff, 2005). *GOGAT* and *GS* isoenzymes play a precise role at specific stages in life cycle of the plant and under explicit environmental conditions linked to mode of N nutrition (Table 1.2). The reason being the differential mode of expression of genes either on the transcriptional stages or post-transcriptional stages (Cren et al., 1999; Lea & Miflin, 2011; Suzuki & Knaff, 2005) as shown in Figure 1.7.

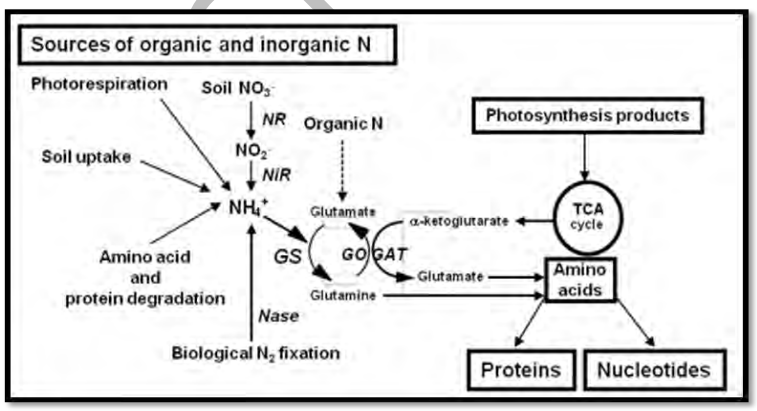

Figure 1.7. The reactions occurring in nitrogen assimilation in higher plants. Nitrate (NO3−), Nitrite (NO2−), Ammonium (NH4+), Atmospheric Dinitrogen (N2)(Hirel et al., 2011).

The nitrogen remobilization is vital for the grain protein content (GPC) of seeds. The nitrogen concentration of seeds influences germination efficiency of seed and the young seedlings persistence efficiency. The nitrogen uptake and assimilation in the grain filling

duration is insufficient for the seed's requirements, so nitrogen is provided to the seeds via various sequentially occurring remobilization steps in different plant organs. The N remobilization from leaf to grain in wheat, rice and maize is cultivar dependent and varies between 50 to 90 percent (MASCLAUX et al., 2001). N remobilization is environment dependent and also favoured during limiting nitrate supplies (Lemaître et al., 2008).

Table 1.2. List of enzymes involved in nitrogen pathway of plants.

Enzyme name	Enzyme abbreviation	Function
Asparagine synthetase	AS	Convert aspartate into asparagine
Aspartate aminotransferase	AST	Convert glutamate into aspartate
Glutamate dehydrogenase	GDH	Dehydrogenate α-ketoglutarate
Glutamine synthase	GS	part of GS/GOGAT cycle
Glutamate decarboxylase	GAD	Glutamate decarboxylation into gamma aminobutyric acid
Glutamine oxoglutarate aminotransferase	GOGAT	part of GS/GOGAT cycle
Nitrite reductase	NiR	convert nitrite into ammonium ion
Nitrate reductase	NR	Convert nitrate into nitrite

1.11. Strategies to understand and improve nitrogen response in plants

1.11.1. Molecular breeding for nitrogen response in plants

Plant breeding has enhanced crop improvement by integration of latest innovations in the field of genetics and biology. Domestication of crop varieties is done by prehistoric selection on the basis of phenotypes that increased productivity (Jain, 1993). In conventional plant breeding, main constraints in phenotypic selection are difficulty in measuring phenotypes for specific trait or identification of individual with maximum breeding value. Moreover conventional plant breeding requires more expense and time. Molecular markers in addition to high-throughput genome sequencing dramatically increased the knowledge about characterization of genetic diversity in germplasm pool of important crop species (Cooper et al., 2004; Elshire et al., 2011; Niebur et al., 2004).

To identify the key regulatory genes involved in multifaceted physiological and agronomic trait's functioning and studied responses of plants to the environmental problems, quantitative genetics has become a very important method by QTL detection during the last few decades (Xu, 1997). The genetic significance of QTL's can be estimated through establishment of QTL's co-location for physiological and biochemical traits with candidate genes (involved in the control of trait of interest) after the location of QTL for phenotypic and

agronomic traits. The validation of candidate genes is then performed either by using forward genetics (transgenic technologies) and reverse genetics (mutagenesis) or by the understanding the relationship between allelic polymorphism and association mapping (trait of interest) either at single level gene or genome wide (Yu & Buckler, 2006). In case of large mapping population, dense genetic marker maps can be used to analyse the contribution of genome's discrete regions. Traits important from agronomical point of view like yield, nutritional quality, durable resistance and flower time which follow polygenic and complex patterns of inheritance in which multiple genes have small effects on the trait value can be analysed by help of markers (Frary et al., 2000; Thornsberry et al., 2001).

Single nucleotide polymorphisms (SNPs), are used commonly for the genotyping of wheat (Akhunov et al., 2009; van Poecke et al., 2013). The KASP assays and high-density iSelect array both are used for uptake of SNP markers in recent years (Allen et al., 2011; Wang et al., 2014). However, in current hexaploid SNP resources, most of the SNP markers developed up-till now are not appropriate to use properly in wide crosses. Due to sequence polymorphism that occurs between bread wheat (hexaploid) and its wild relatives is a problem for designing for array-based PCR primers. In order to solve said problem, Wang et al. (2014), used a platform that is array-based and through which it is easy to examine and authenticate more than 81,000 putative SNPs in both hexaploid and tetraploid wheat.

1.11.2. Association mapping

Association mapping (AM) or association analysis is an innovative methodology which complements QTL analysis. It is one of the important tools for molecular plant breeding through which, gene effects are detected on a linkage disequilibrium (LD) basis (Breseghello & Sorrells, 2006). As both these terms, LD and AM are interchangeably used but there are understated differences between them. According to (Gupta et al., 2005). LD is non-random association between 2 genes or 2 markers whilst AM is referred to as marker locus substantial association to the phenotype traits. Thus, in other words AM is LD application. However, tight linkages of alleles present on same chromosome translate mostly into high Linkage Disequilibrium. Significant LD can be observed between distant loci (Soto-Cerda & Cloutier, 2012).

Many methodologies have been developed for AM and some of those are perfectly applicable either with or without modifications for wide range species like plants. Pritchard et

al. (2000) developed a structure association (SA) analysis that used randomly selected markers at first for Q-matrix (Population Structure) estimation and then for the rectifications of false association, this estimation was incorporated into a general linear model (GLM). Another model MLM (Mixed Linear Model) was established by Yu and Buckler (2006) that also incorporates K-matrix/kinship i.e., familial-relatedness along with population structure.

Genome-wide association mapping is a popular method which identifies quantitative trait locus (QTL) for large number of crops including wheat (*Triticum aestivum L.*). It has an edge over traditional bi-parental mapping strategies that depend on the degree of linkage disequilibrium (LD) in the mapping population (Edae et al., 2014). The development of new statistical approaches along with novel molecular markers for a wide range of dense genomic coverage for association mapping (AM) permits identification genetics of a trait in a better way (Lorenz et al., 2011). The entire genome is more precisely explored by using different association mapping methods (Bordes et al., 2014). Association mapping requires densely genotyped population with significant genetic variability for concerned traits. Different association panels are used in wheat (*Triticum aestivum L.*) for identification of loci controlling agronomic (Breseghello & Sorrells, 2006; Crossa et al., 2007) and quality (Bordes et al., 2011; Ravel et al., 2009) traits in several association mapping (AM) studies.

1.11.3. QTLs for nitrogen responsiveness in wheat

Quantitative trait loci for the uptake efficiency of nitrogen and activities of nitrogen enzyme in Wheat have been explored recently (Fontaine et al., 2009; Habash et al., 2007; Laperche et al., 2007). Habash et al., (2007) unvaryingly co-localized QTLs for GS activity with those for grain nitrogen and found that high GS activity is linked with high nitrogen in grain. The outcome for this research was confirmed through another population by Fontaine et al. (2009). On the other hand, wheat crop did not show any correlation unlike maize crop. Quraishi et al., (2011) provided a complete view of NUE meta-QTL by describing the first integration of known QTLs. In this study, the meta-analysis methodology was executed by using synteny-based physical mapping and cross-genome comparison. Meta QTLs for NUE were mapped on chromosome 3B of Wheat through comparison with previous literature having NUE identified QTLs for other cereal genomes such as rice, sorghum, and maize. Mapping of an ortho-meta-QTL was performed using the consensus markers across 4 genomes to increase the accuracy and precision of detecting QTL then ultimately candidate gene identified

responsible for Bread Wheat NUE was an NADH-GOGAT gene. All in all, GOGAT gene is suggested as gene driving NUE evolution via an ancestral proto chromosomal locus due to various events of sequence shuffling (Quraishi et al., 2011).

In literature, to date fifteen studies have been investigated the N responsive genomic regions in bread wheat (Table 1.3). These independent studies had reported QTLs in wheat under N stress for important agronomic traits (An et al., 2006b; Brasier et al., 2020; Deng et al., 2017; Fan et al., 2019; Fan et al., 2018; Fontaine et al., 2009; Guo et al., 2012; Habash et al., 2007; Laperche et al., 2016; Sun et al., 2013; Xu et al., 2014; Zhang et al., 2019).

Table 1.3. QTL studies for nitrogen responsive and related traits in wheat, source: (Saini et al., 2021)

Type, cross (Size) [no. of environments]	Marker types[a] (number of markers)	N QTLs	Reference
DH, Hanxuan 10/Lumai 14 (120) [2]	AFLP, SSR, and EST (395)	33	An et al. (2006)
DH, Arche/Re´cital (120) [1]	SSR and gene specific markers such as Glu loci, SPA, Rht loci, and Fdgogat-D1 (188)	32	Laperche et al. (2006)
DH, CS/SQ1 (91) [1]	SSR and others (449)	164	Habash et al. (2007)
DH, Arche/Re´cital (222) [8]	SSR and gene specific markers such as Glu loci, SPA, Rht loci and Fdgogat-D1 (188)	43	Laperche et al. (2007)
DH, Arche/Re´cital (222) [6]	SSR and gene specific markers such as Glu loci, SPA, Rht loci and Fdgogat-D1 (188)	35	Laperche et al. (2008)
DH, Arche/Re´cital (137-221) [3]	SSR and gene specific markers such as Glu loci, SPA, Rht loci and Fdgogat-D1 (197)	157	Fontaine et al. (2009)
RILs, Chuan 35050/Shannong 483 (131) [12	DArTs, SSRs, EST-SSRs and biochemical markers (719)	192	Guo et al. (2012)
RILs, Chuan 35050/Shannong 483 (131) [3]	DArTs, SSRs, EST-SSRs and biochemical markers (719)	148	Sun et al. (2013)
RILs, Xiaoyan 54/Jing 411 (182) [6]	SSR, EST-SSR, and Glu loci (555)	48	Xu et al. (2014)
DH, RAC875/Kukri (148-156) [18]	SSR, DArTs, and SNP (1333)	28	Mahjourimajd et al. (2016)
DH, Huapei 3/Yumai 57 (168) [4]	SSR, EST, ISSR, and HMW-GS (323)	69	Deng et al. (2017)
RIL, Kenong 9204/Jing 411 (188) [3]	SNPs, SSR, EST-SSR, ISSR, STS, SRAP and DArT (119,566)	62	Fan et al. (2018)
RIL, Kenong 9204/Jing 411 (188)	SNPs, SSR, EST-SSR, ISSR, STS, SRAP and DArT (119,566)	157	Fan et al. (2019)
RILs, Tainong 18/Linmai 6 (184)	DArT, SNPs, and 105 SSR (5399)	251	Zhang et al. (2019)
RILs, Yorktown/VA05W-151 (136); DH, Yo	SSR and SNPs (3918); SSR and SNPs (3147)	66; 64	Brasier et al. (2020)

1.11.4. Nitrogen responsive genes manipulation

Nitrogen uptake efficiency can be improved using crop varieties that are high in nitrogen efficiency, have high yield and a reduced input of nitrogen (Garnett et al., 2015; Miller et al., 2008; Sanders et al., 2009). It was shown in recent studies that nitrogen metabolism and uptake is influenced by the pathways of shoot to root signalling, feedback mechanism, and transportation of amino acids in shoots and roots (Araus et al., 2016; Fang et al., 2013; Forde & Roberts, 2014; Santiago & Tegeder, 2016; Tan et al., 2010). To improve NUE, many steps have been taken towards genetic changes in nitrite allocation (Chichkova et al., 2001), nitrogen uptake (Ameziane et al., 2000; Chen et al., 2017; Tsay et al., 2011), nitrogen regulation (Ferrario-Mery et al., 1998), and nitrogen metabolism (Habash et al., 2001; Seiffert et al., 2004; Yamaya et al., 2002). Besides, plant nitrogen stress biomass has also been tested by knock out and over expression of several candidate genes. Over expression of *HATS*-like *NRT2.1* resulted in an increase of nitrate influx, but its utilization as well as uptake remained unchanged (Olson et al., 1979). The efficacy of *NR*/*NiR* encoding genes in transgenic plants for the improvement of NUE has no surety at all. Besides, a delayed Nitrate reductase activity was recorded in tobacco plants was shown by *NR*-related genes in tobacco plant during drought conditions, but a quick recovery was observed on re-watering after a short-time drought as well (Hoshida et al., 2000).

A decrease in nitrate level of transgenic Tobacco, Potato, and Arabidopsis plants has also been reported without any improvement in tubers and seed number along with biomass. Overexpression of *Nia*/*Nii* genes also increased the levels of mRNA regardless of the available nitrogen sources. NUE was also affected without any change in growth and yield. This indicates the NR's composite post transcriptional regulation (Migge et al., 2000). Talking about gene expression of *GS1* and *GS2*, overexpression of *GS2* gene has been checked in tobacco plants using *CaMV 35S* or *Rubisco* promoters in *Oryza sativa* (Good et al., 2004; Oliveira et al., 2002). An enhanced drought tolerance and photorespiration was observed in *Oryza sativa* and better growth rate was observed in *Nicotiana tabacum*. Biomass and Grain yield has also showed positive results by overexpression of *GS1* genes having promoter with different combinations like Rubisco subunit (*rbcS*), *CaMV* 35S, and *RolD*. Nitrogen efficient wheat lines having *rbcS* promoter showed higher root length and grain yield with high nitrogen content (Yanagisawa et al., 2004). *Nicotiana tabacum* with over expressed *GS1* having *CaMV* 35S

promoter showed an increased level of total leaf proteins and biomass (Deprost et al., 2007). Maize yield increased 30% with more kernel size and number due to *GS1* gene overexpression (Garnett et al., 2015). All in all, *GS* gene activity is related directly to yield and biomass in transgenic plants (Castaings et al., 2009).

Garnett et al., (2015) reported an increased grain yield of transgenic *Oryza sativa* due to overexpression of *NADH-GOGAT*. It is thus important to recognise the genes, promoters, and alleles for the improvement of yield by *GOGAT*/*GS* genes overexpression. Soluble protein content of seed, ability of plant growth in limited supply of nitrogen, and total proteins increased in Arabidopsis by the overexpression of *ASN1* gene (Potel et al., 2009). All these studies propose that by the manipulation of nitrogen remobilization's downstream steps, NUE can be improved. NUE improvement can also be obtained by further studies on carbon metabolism pathways (Lam et al., 1998; Lea et al., 2006; Pathak et al., 2008). Expression of genes that are regulated highly at transcriptional and post-transcriptional levels is greatly influenced by both endogenous and external factors (Meyer & Stitt, 2001). Yamaya et al. (2002), reported that levels of ammonium, amino acids, and nitrate are affected by post-translational regulation, whereas only a minor influence was observed in case of transcriptional regulation. Besides, higher concentration of glutamine and asparagine accumulation was observed in leaves of plants that are unregulated for NR. Formation of asparagine (*Asn*) is catalysed by a small gene family which encodes Asparagine synthetase (AS). It also catalysed Glutamate formation from Glutamine (*Gln*) and aspartate (Harrison et al., 2000).

The interaction role of AS and GS in primary metabolism of nitrogen is very important (Carvalho et al., 2003; Harrison et al., 2003). The negative correlation of GS with polypeptides and transcript levels of AS suggest that compensation of GS ammonium assimilatory activity is showed by AS (Harrison et al., 2003; Wong et al., 2004). It is considered that due to the decrease of GS activity in plants, AS might be very important in reduced N flux regulation. For the biosynthesis of Asp using AspAT and NADH-GOGAT, it is however important to synthesize Gln for which, GS is vital (Harrison et al., 2003). Potel et al. (2009) reported that overexpression of *ASN1* gene in Arabidopsis enhanced growth on nitrogen limited medium with improved total protein content, and soluble seed protein. However, accumulation of endogenous ammonium was greater in plants grown on 50 mM ammonium medium as compared to wild type in case of *ASN2* gene (Moose & Below, 2009). For metabolic engineering, signalling processes are attractive clues. GDH (glutamate dehydrogenase)

physiological activity is still not clear compared to GOGAT/GS enzymes (Dubois et al., 2003). GDH activity was investigated by Ameziane et al. (2000) in tobacco transgenic plants, where biomass production increased in case of GDH transgenic plants irrespective of controlled and field conditions.

1.11.5. Microarray and whole genome sequencing

The hypothesis that NRE capacity is improved by conventional breeding is supported by the fact that nitrogen utilization enhanced but nitrogen uptake remains constant throughout the domestication of maize varieties (Hirel et al., 2007a). Interestingly, for the improvement of phenotypic change or NUE, inconsistency of over expressed key enzymes like NiR, GOGAT, NR, and GS is also a challenge (Castaings et al., 2009; Garnett et al., 2015; Hirel et al., 2007a; Yamaya et al., 2002). New molecular techniques like transcriptome and microarray due to this reason are now considered as emerging tools for the study of plant whole genome response (Figure 1.8).

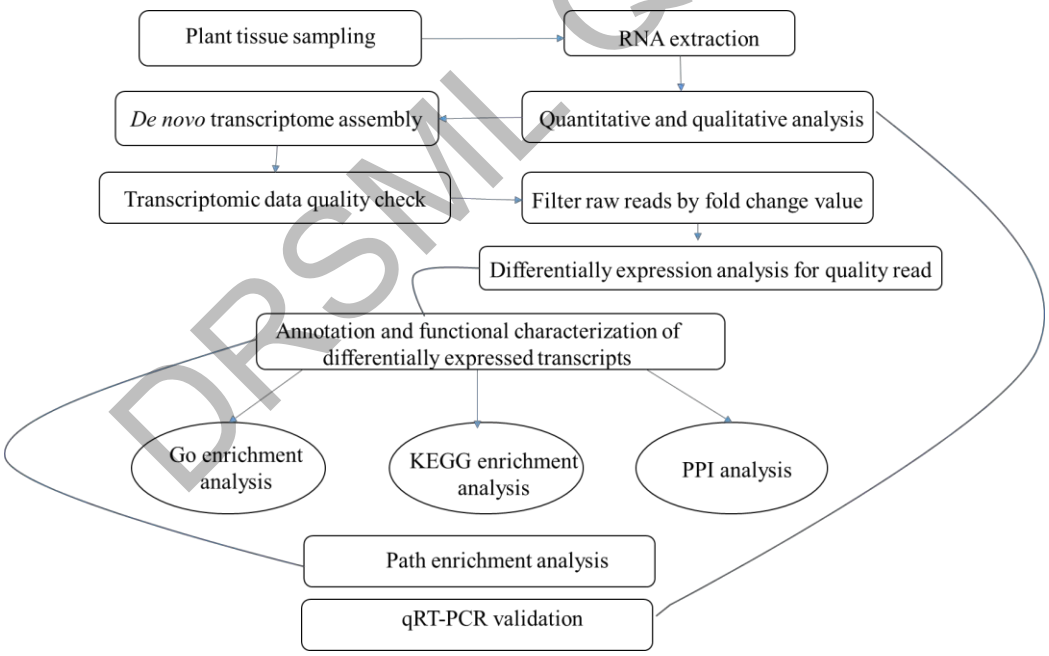

Figure 1.8. Workflow chart for transcriptomic profiling for crops (Anas et al., 2020).

Microarray is the arrangement of both unknown and known DNA samples on a solid support. Every microarray contains thousands of probes that are a spot with less than 200 µM dm (Radtkey et al., 2000). These arrays could be in various formats and the probes can be as small as cDNA, genomic sequences, and oligonucleotides. Various techniques employed to the

format are nib, inkjet, pin, and photolithographic. The labelling of these probes is achieved through hybridization monitored electronically, fluorescently, or radioactively (Wang et al., 2009).

To recognise the changes at gene expression level, genomic level, and specific genes related to desired traits, the modern approach is whole genome sequencing. Transcriptomic profiling is an excellent emerging technique for whole genome sequencing of all plants. In case of Arabidopsis and ideotype rice, good quality genome sequence information is available for microarray analysis (Figure 1.8) (Lian et al., 2006; Rawal et al., 2017). Differently expressed genes (DEG's) are known using the physiological and molecular techniques for low levels of nitrogen in last two decades in *Oryza sativa* (Gelli et al., 2014; Yang et al., 2015), *Glycin max* (Li et al., 2017), *Camilia sinensis* (Cho et al., 2007), and *Sorghum bicolor* (Hao et al., 2011).

Previous studies globally relied on single genotype for the expression of genes for either ammonium or nitrate in case of low and normal nitrogen (Cho et al., 2007; Gelli et al., 2014; Hao et al., 2011; Li et al., 2017; Yang et al., 2015). Two genotypes were studied at both levels of ammonium and nitrogen form in *Camilia sinensis.* The knowledge for NUE candidate gene was compacted using the comparative analysis and global genes expression of genotypic contrast. Besides, lots of QTL (Quantitative trait loci) related literature associated with NUE is also available (Pandit et al., 2010; Wei et al., 2012; Zhou et al., 2017). In future, for the development of new NUE genotypes, QTLs and DEGs dataset combination is considered very important (Curci et al., 2018).

For the understanding of transcript regulation and gene transcription at all levels, next generation sequencing (NGS) technologies to develop transcriptomic profiles are very useful (Wan et al., 2017). The plant's response to nitrogen nutritional stress was investigated using Illumina's RNA-sequencing platform. Amino-acid transporters (AAT) play a significant role in transportation of N under abiotic stress at different developmental stages. The wheat grain regulatory mechanism for storage protein in response to nitrogen supply during development of grain based on transcriptomic profiling. Asparagine is considered as an ideal transporting molecule of nitrogen as it is very important for uptake of nitrogen in roots (Harrison et al., 2000; Kirkman & Miflin, 1979; Todd et al., 2008). According to Curci et al. (2018), under the limited nitrogen stress, asparagine encoding genes were downregulated in both roots and leaves of durum wheat (Wan et al., 2017). The plants that were grown under nitrogen free conditions

showed down regulation of genes in both leaves and roots that were involved in amino acids, nitrogen, and carbon metabolism along with photosynthetic activities (Gelli et al., 2014).

1.12. Aims and objectives of the study

The general aim of the present study was to evaluate the nitrogen response in historical bread wheat panel with different omics approaches, to identify quantitative trait loci associated with the N related traits, computed statistical investigation to decrypt the contribution of grain yield components and root traits towards the final grain yield in wheat under high and low nitrogen application, assess the wheat varietal response to mitigate terminal heat stress under variable N application regimes, and determine how related *NAM* genes control nitrogen remobilization at the molecular level in bread wheat.

Specific aims of each chapter were:

- ***Chapter#2:*** The objective of the study was to unravel the genetic basis behind the nitrogen response using 90K SNP array by GLM, MLM, and FarmCPU based Genome Wide Association Mapping in a diverse panel comprising landraces, green revolution, post green revolution, elite cultivars, and CIMMYT advance cultivars.
- ***Chapter#3:*** The study aimed to examine the major grain yield components and root traits and their level of contribution for yield maximization under variable N supplies through multiple linear regression and building their path model using LISREL software. It computes multiple linear regression (MLR) to show the interaction between independent (grain yield components and root traits) and dependent (grain yield) variables in the form of direct effect (DE), indirect effect (IE) and total effect (TE).
- ***Chapter#4:*** The objective of the study was to demonstrate the wheat varietal response to RSI and RNDVI at the anthesis stage and their relationship to yield and yield-related traits under variable N supply and terminal heat stress. This work will help the researchers to identify and develop nitrogen-use efficient and thermos-tolerant wheat cultivars by minimizing the negative impacts of heat stress at the anthesis stage.
- ***Chapter#5:*** The study aimed to address the lack of time-resolved understanding of *NAM* gene regulation of senescence and nutrient remobilisation, we analysed flag leaf tissues at seven time points from wild type and *NAM* RNAi wheat plants. We characterised gene expression changes in nitrogen-associated genes during senescence in wild type and *NAM* RNAi plants and identified genes through which *NAM* genes may influence nitrogen remobilisation.

Chapter #2

Genome-Wide Association Study of Nitrogen Response in Triticum aestivum L.

Chapter # 2

Genome-Wide Association Study of Nitrogen Response in *Triticum aestivum L.*

2.1. Abstract

Nitrogen (N) fertilizer plays a significant role in wheat grain yield potential and quality. Excessive use of nitrogen fertilizer pollutes the environment and raises production costs. Efficient N use is critical for sustainable agriculture. To detect marker-trait associations (MTAs) related to complex nitrogen linked agronomic traits, field experiments over two consecutive years (2016-17 and 2017-18) were conducted on 124 wheat varieties under three different nitrogen application rates: control (C; 120kgN/h), treatment 1 (T1; 79.2 kg/h), and treatment 2 (T2; 39.6 kg/h). There was significant phenotypic heterogeneity across treatments and seasons for all ten agro-physiological traits including chlorophyll content (CHL), normalized difference vegetation index (NDVI), flag leaf area (FLA), tiller per plant (T.P), plant height (PH), biomass (BM), grain yield (GY), grain per spike (GpS), harvest index (HI) and nitrogen agronomic efficiency (NAE) assessed in this study. Grain yield and agro-physiological traits were shown to be significantly positively correlated. Using 20,853 single nucleotide polymorphism markers across the wheat genome, 1412 MTAs at $-\log_{10}P > 3.0$ related to ten agro-physiological traits under study at varying N levels (C, T1, and T2) were found. Of these, 540 MTAs for 9 traits in the control (C), 479 MTAs for 10 traits in the treatment 1 (T1), and 393 MTAs for 10 traits in the treatment 2 (T2) were detected. A genome-wide association study (GWAS) identified 274 significant marker trait associations (MTAs) at $-\log_{10}P > 3.7$, of which 72 were identified by two or three methods including FarmCPU (Fixed Random Model Circulating Probability Unification), MLM (Mixed Linear Model), and General Linear Model (GLM). These 72 significant MTAs verified by more than one method were selected for gene identification conferring chlorophyll content, normalized difference vegetation index, flag leaf area, plant height, tiller number, grain yield, biomass, harvest index, grains per spike and nitrogen agronomic efficiency. Genes corresponding to the significant MTAs were retrieved as candidate genes, including members of the transcription factor families and protein kinases. Identified putative candidate genes associated with significant MTAs, may be directly or indirectly involved with various biological processes, molecular functions and cellular component organization. These candidate genes might also play key roles in plant growth and development along with grain production.

2.2. Introduction

The demand for nitrogen at global level is currently up to 117 million metric tonnes, with an expected 1.5% increase annually in the coming years (FAO, 2019). Farmers typically use high nitrogenous fertilizer rates to ensure high yields. The excessive use of commercially available fertilizers has resulted in deterioration of air, soil, and water quality (Hickman et al., 2014; Russo et al., 2017). Furthermore, when the supply of nitrogen (N) exceeds crop N demand, plants become more susceptible to various diseases and insect pests (Reddy, 2017). As a result, it is critical to optimize and improve cereal crop nitrogen use efficiency (NUE) in order to maximize yield while minimizing the negative impact of increased N use on the environment and natural resources. Identification of marker-trait associations (MTAs) can be applied to make significant tailored introgressions and is one potential genetic method for addressing the challenge of developing N-efficient wheat cultivars with stable output in N-limited environments.

Wheat cultivars that can sustain yield under the application of moderate or severe N deficient conditions can adapt to low N input systems in a better way. Genetic variation for adaptation traits to N deficiency is required to breed such varieties. To date, only a few quantitative trait loci (QTL) for yield and its response to N deficiency in wheat have been identified under field conditions. A variety of genetic loci for agronomic traits linked to N use and grain yield in wheat and rice have also been mapped to the chromosomal regions containing the GS2 gene (Fontaine et al., 2007; Laperche et al., 2008; Obara et al., 2004; Prasad et al., 1999). This suggests that the genomic region surrounding *GS2* can help in the development of wheat and rice cultivars with improved agronomic efficiency and nitrogen response (Pritchard et al., 2010). Other genetic regions associated with N uptake in wheat (Su et al., 2006), maize (Zhu et al., 2005), rice (Ming et al., 2000; Wissuwa et al., 1998), common bean (Liao et al., 2004), and soybean (Liang et al., 2010) have also been identified.

In the present study, we have used an alternative method to assess the nitrogen (N) status of wheat crop which is more efficient and farmer friendly. Various studies reviewed by Ali et al., (2017) have shown that nitrogen status of crops or plants can be diagnosed through leaf chlorophyll content. One of most instantaneous and non-destructive method for chlorophyll content measurement can be Minolta SPAD meter. SPAD readings have direct correlation with leaf chlorophyll content at specific growth stages in various plant species (Peng et al., 1993) including *Oryza sativa L.* (Yuan et al., 2016), *Zea Mays L.* (Ziadi et al., 2008), *Triticum aestivum L.* (Arregui et al., 2006). Consequently, SPAD meter is recognized

as a tool used for detection and monitoring of N status and deficiencies in plants by comparison of CM (chlorophyll meter) readings of fully nitrogen fertilized treatment with other N treatments (Blackmer & Schepers, 1995; Vidal et al., 1999). Another alternative and effective approach to identify crop N status is the use of GreenSeeker sensors which have been reported in many published studies for detection of crops' nitrogen status (Arnall et al., 2006; Freeman et al., 2007a; Raun et al., 2002). Many producers have reported an improvement of 15% N fertilizer utilization of cereal crops by use of an efficient GreenSeeker.

Quantitative trait loci (QTL) mapping can elucidate the molecular basis of complex traits using high throughput genotyping and phenotyping datasets (Langridge & Reynolds, 2015). Among QTL mapping methods, genome-wide association study (GWAS) has the upper hand because it provides higher QTL mapping resolution and investigates all evolutionary recombination events (Flint-Garcia et al., 2003; Yu & Buckler, 2006). Simple sequence repeats, restriction fragment length polymorphism, expressed sequence tags, amplified fragment length polymorphism, the diversity array technique, random amplified polymorphic DNA and single nucleotide polymorphism markers have all been used for QTL mapping. For GWAS, the TASSEL, PLINK, GAPIT, EMMAX, GenABEL, GEMMA, FarmCPU pkg and GCTA packages were used to run FarmCPU (Fixed Random Model Circulating Probability Unification), MLM (Mixed Linear Model), and General Linear Model (GLM) to identify significant marker trait associations (Aulchenko et al., 2007; Bradbury et al., 2007; Kang et al., 2010; Lipka et al., 2012; Purcell et al., 2007; Tang et al., 2016; Yang et al., 2011; Yin et al., 2021; Zhou & Stephens, 2012). The computational analysis using these packages has become more complicated when the number of samples and SNPs in GWAS has increased. The Memory-efficient Visualization-enhanced Parallel-accelerated (rMVP) package has been developed to improve computational efficiency. It processes large data sets effectively, estimates population structure in an efficient manner, evaluates variance components more rapidly, and utilizes GLM, MLM, and FarmCPU analysis methods to identify marker trait associations (Yin et al., 2021).

In the present study, genome-wide association study (GWAS) assessed a set of 124 historical bread wheat varieties of Pakistan using high-density SNP markers array for agro-physiological traits under three N fertilization regimes in the field. GWAS was used to identify MTAs for the agro-physiological traits, and identified candidate genes underlying the nitrogen related agro-physiological trait in wheat which provides a basis for future breeding to improved N response.

2.3. Materials and methods

2.3.1. Plant material

A diverse historical panel of 124 Pakistani bread wheat cultivars including landraces, green revolution, post green revolution, and elite cultivars adapted to different climatic zones (irrigated, semi-arid, and arid; Appendix 2.1) were used in this study. The seeds of the selected cultivars were obtained from Wheat Wide Crosses Laboratory, National Agricultural Research Centre, Islamabad, Pakistan.

2.3.2. Field experiment

The selected association panel was subjected to field trials for two consecutive cropping seasons from 2016 to 2018 at the National Agricultural Research Centre, Islamabad, Pakistan located between 33°40'28"N latitude and 73°7'28"E longitude. Planting was done on November 15 each year in an alpha lattice design. Each plot consisted of four 1 m rows with a sowing density of 20 seeds per row and spaced 20 cm apart from adjoining plots. The field trials were managed by standard agronomic practices.

2.3.3. Phenotyping

Agro-physiological traits including chlorophyll content (CHL), normalized difference vegetation index (NDVI), flag leaf area (FLA), tiller per plant (T.P), plant height (PH), biomass (BM), grain yield (GY), grain per spike (GpS), harvest index (HI) and nitrogen agronomic efficiency (NAE) under three different nitrogen application rates: control (C; 120kgN/h), treatment 1 (T1; 79.2 kg/h), and treatment 2 (T2; 39.6 kg/h) were recorded. All the traits were measured according to the procedures described by Pask et al. (2012).

Chlorophyll content was measured from 1/3 of the distance, 1/2 of the distance, and 2/3 of the distance from base of the flag leaves of three central plants for each genotype between 11am and 3pm. The average of nine readings from three replicates at each time point was used for further analysis. Normalize Difference Vegetative Index (NDVI) was recorded at heading, anthesis, 14 DAA (mid grain filling duration) between 11 am and 2 pm by measuring the canopy reflectance at 660 nm and 770 nm [(R770-R660)/(R770+R660)] with a handheld GreenSeeker crop sensor (Trimble). The distance between the canopy and the NDVI meter was kept around 50 cm. Plant height (PH) was assessed by measuring the plant from base to tip of the spike excluding awn using a measuring rod at physiological maturity. Tillers per plant (TP) were recorded by counting the total number of fertile tillers in individual plant at anthesis.

Spike length (SL) was determined by measuring the spike from base of the rachis to tip of the upper spikelet, excluding awns at physiological maturity. The average values from three biological replicates for PH, TP and GpS were used for statistical analysis. The above ground biomass excluding row edges was harvested, dried, and weighed using an electronic balance to determine biomass (BM). The harvested above ground biomass was threshed and grain harvest obtained after threshing was weighed using an electronic balance to measure grain yield (GY).

2.3.4. Statistical analysis

Phenotypic data was subjected to best linear unbiased predictions (BLUPs) analysis using lme4 package in R version 3.5.1 (Bates et al., 2015). BLUPs estimate the real breeding value of a trait by eliminating environmental anomalies (Robinson, 1991; Viana et al., 2010; Mi et al., 2011). BLUPs data for each trait was used for descriptive statistics, and correlation analysis. Analysis of variance (ANOVA) was performed on primary five years field data for each trait. Descriptive statistics and ANOVA were performed by XLSTAT version 2014.5.03. Trait correlations were analyzed and visualized using GGally package in R version 3.5.1.

2.3.5. Genotyping

The genomic DNA was extracted from fresh leaves of 25 days old wheat seedlings according to the CIMMYT Molecular Genetics Manual (Dreisigacker et al., 2012). The DNA with 50-100 ng/µL concentration per sample was sent to CapitalBio® genotyping facility in Beijing for genotyping via high-density Illumina 90K Infinium SNP array consisting of 81,587 markers (Akhunov et al., 2009; Wang et al., 2014). Genome Studio program version 2011.1 was used for genotype calling. Genetic similarities were estimated by PowerMarker v.3.0 with Dice coefficient based on ratio of shared alleles (Liu et al., 2005). Polymorphism information content was employed to determine genetic diversity at each chromosomal locus. Monomorphic markers, markers having missing values more than 20% or allele frequency less than 5% or an unclear SNP calling were removed. The effective 20,853 SNP markers were used for estimation of population structure analysis, principal component analysis, kinship analysis, and genome wide association mapping. The International Wheat Genome Sequence Consortium reference assembly (IWGSC) RefSeq-v.1.0 was used to determine physical positions of SNP markers along chromosomes.

2.3.6. Population structure

Population structure was determined using STRUCTURE software 2.3.3, which uses model-based Bayesian cluster analysis. A total of 1000 unlinked SNP markers, 100,000 burns in iterations followed by 500,000 Markov- Chain iteration were used to give a putative number of subpopulation between k= 1 to 15 (Pritchard et al., 2000). Sampling variance was estimated by 10 independent runs for each k. The rate of change of log probability between the successive values basis for ΔK was used to estimate K (Evanno et al., 2005; Quraishi et al., 2011).

2.3.7. Linkage disequilibrium, genome wide association analysis and gene annotation

The observed allele frequency and expected allele frequency were used to calculate linkage disequilibrium (LD) in TASSEL v.5.0. The Memory efficient Visualization enhanced and Parallel accelerated (rMVP) R package with default setting was used for Genome Wide Association Study (GWAS). The rMVP employed three models i.e., General Linear Model (GLM), Mixed Linear Model (MLM), and Fixed and random model Circulating Probability Unification (FarmCPU) to estimate the marker trait associations (MTA). For multiple testing correction, the bonferroni correction was applied to calculate the threshold. The association between marker and trait was considered significant if the –log10 (p) value was greater than the threshold of –log10 (p) >3.7. Finally, genes associated with the locus were extracted from *Triticum aestivum* genes (*IWGSC*) dataset at *Ensembl Plants* using BioMart function.

2.4. Results

2.4.1. Phenotyping analysis and relationship among traits

We evaluated agro-physiological traits of a historical bread wheat panel of Pakistan, for two years and BLUPs data. For all ten traits, the effect of varieties was significant, depicting the noticeable genetic variation across the whole germplasm. Additionally, the effect of treatments (N-levels) were highly significant at 0.001 for all agro-physiological traits except for FLA (0.01**), PH (0.007**) and GPS (0.009**) which were comparatively less significant at 0.01. The N-level x varieties interaction effect was not highly significant for all studied traits (Table 2.1).

Nitrogen fertilization had most significant effects on all traits under study. All traits showed highest maximum range under control (N=120 kg/ha) followed by T1 (N=79.3 kg/ha) and T2 (N=39.6 kg/ha) respectively. The largest differences between C, T1 and T2 was observed for GY, BM, CHL and NDVI, probably these traits strongly depend on theoretically available N in soil. Other traits such as FLA, PH and PH showed significant but moderate response to nitrogen level. While at T2, significant reduction in GY, GpS, TP, NAE and CHL in majority of varieties (Table 2.1).

Correlation test was performed for all agro-physiological traits (Figure 2.1) under three different nitrogen application rates: control (C; 120kgN/h), treatment 1 (T1; 79.2 kg/h), and treatment 2 (T2; 39.6 kg/h). Significant correlation was observed among different traits. Under control, GY showed significant positive correlation with FLA at r=0.281*. Correlations of CHL with some important agronomic traits was significantly positive in T1 including NDVI (r=0.422***), BY (r=0.227*), GY (r=0.262*) and GpS (r=0.228*; Figure 2.1). Under control application of N fertilizer most of the traits showed non-significant correlation GY and BM. NDVI showed significant correlation with all traits including FLA, BM, GY, GpS except T.P, PH and NAE under T1. Under minimum application of N fertilizer i.e. T2, FLA is negatively and significantly correlated with T.P with r=-0.377*(Figure 2.1). Under treatment 2 (T2), many traits showed a correlation which was weak and not significant (Figure 2.1).

Table 2.1 . Descriptive statistics and analysis of variance, of the agrophysiological traits evaluated for Pakistan historical bread wheat panel under three different nitrogen application rates: control (C; 120kgN/h), treatment 1 (T1; 79.2 kg/h) and treatment 2 (T2; 39.6 kg/h).

Trait	Year	C		T1		T2		ANOVA (p value)		
		Range Units?	**Mean±std. Deviation**	**Range** Units?	**Mean±std. Deviation**	**Range** Units?	**Mean±std. Deviation**	**N-levels**	**Varieties**	**Interaction**
CHL	2016-17	26.4~55.37	42.01±5.6	22.07~50.47	40.09±5.78	22.5~53.8	38.38±6.3	0.002**	0.544	0.874
	2017-18	35.47~56.3	46.73±4.35	30.64~54.3	43.93±4.68	30.77~53.4	41.34±4.14			
	BLUPs	30.46~61.07	44.55±6.84	20.47~59.03	41.71±7.93	21.85~56.8	40.01±6.93			
NDVI	2016-17	0.49~0.78	0.67±0.05	0.38~0.73	0.63±0.07	0.41~0.72	0.61±0.05	0.001***	0.043*	0.487
	2017-18	0.66~0.8	0.74±0.04	0.5~0.77	0.7±0.05	0.5~0.77	0.69±0.06			
	BLUPs	0.62~0.79	0.71±0.04	0.37~0.8	0.66±0.1	0.46~0.74	0.64±0.07			
FLA	2016-17	26.31~77.95	40.63±7.7	20.99~59.82	35.35±7.74	16.97~64.17	33.06±9.07	0.01**	0.733	0.735
	2017-18	23.02~81.95	41.08±7.46	15.99~57.82	36.02±8.11	13.58~59.17	30.69±8.84			
	BLUPs	21.15~68.35	41.89±9.81	15.66~67.9	36.79±11.3	11.97~67.89	33.03±12.94			
TP	2016-17	4~11	5.58±2.07	2~7	4.96±1.3	3~7	4.66±1.15	0.003**	0.483	0.446
	2017-18	2~11	5.81±2	2~11	5.32±1.82	2~8	4.38±1.48			
	BLUPs	3.35~9.77	5.7±1.54	3.45~8.6	5.46±1.21	2.95~8.07	5.27±1.17			
PH	2016-17	86.57~131.74	104.16±7.53	75.57~129.04	99.72±7.95	74.04~118.47	99.78±7.38	0.007**	0.869	0.875
	2017-18	66~138.3	103.58±12.84	55.8~130.2	98.7±11.66	53~120.5	93.08±9.41			
	BLUPs	66.54~137.56	103.2±12.96	69.14~136.71	98.13±14.55	53.2~117.24	95.49±11.64			
BM	2016-17	372.9~1303	663.33±143.01	125~1330	551.31±151.07	205~715	493.1±125.5	0.001***	0.434	0.536
	2017-18	314.7~1295	671.13±145.14	119~857	540.51±125.06	199~709	490.12±119.61			
	BLUPs	139.2~1307.51	691.53±237.94	128.69~1097.66	545.12±216.44	77.27~908.64	482.96±222.05			
GY	2016-17	83.06~347.36	201.13±49.79	50.73~248.36	140.84±41.94	37.14~187	111.09±34.77	0.001***	0.527	0.534
	2017-18	90.06~338.36	197.03±48.27	52.64~303.01	139.46±41.82	37.95~310	121.48±48.96			
	BLUPs	49.58~364.21	203.75±65.13	48.82~318.28	143.81±56.2	40.37~264.76	114.08±52.07			
GpS	2016-17	36~77	58.47±9.89	23~75	51.59±11.54	17~76	47.92±12.83	0.009**	0.752	0.935
	2017-18	31~79	58.13±10.81	25~77	51.2±11.9	12~75	46.16±13.25			
	BLUPs	23.69~77.11	57.56±11.26	22.43~69.49	45.8±11.33	17.89~69.38	42.14±13.05			
HI	2016-17	12.21~57.98	31.15±8.67	8.53~46.13	25.6±6.54	7.24~45.71	22.95±6.28	0.001***	0.322	0.361
	2017-18	13.13~59.47	30.16±8.05	11.85~44.65	25.53±6.35	8.24~49.71	24.7±8.08			
	BLUPs	10.26~62.39	29.62±11.25	9.48~60.71	25.95±10.18	7.74~44.32	22.87±6.82			
NAE	2016-17			0.01~1.94	0.77±0.39	0.18~5.56	2.28±1.1	0.001***	0.732	0.794
	2017-18			0.05~1.73	0.73±0.37	0.11~4.53	1.91±1.08			
	BLUPs			0.06~2.34	0.81±0.54	0.09~6.03	2.29±1.54			

Significant values: *** < 0.001, ** < 0.01, * < 0.05, ns > 0.05. Abbreviations: Chlorophyll content (CHL), normalized difference vegetation index (NDVI), flag leaf area (FLA), tiller per plant (T.P), plant height (PH), biomass (BM), grain yield (GY), grain per spike (GpS), harvest index (HI) and nitrogen agronomic efficiency (NAE).

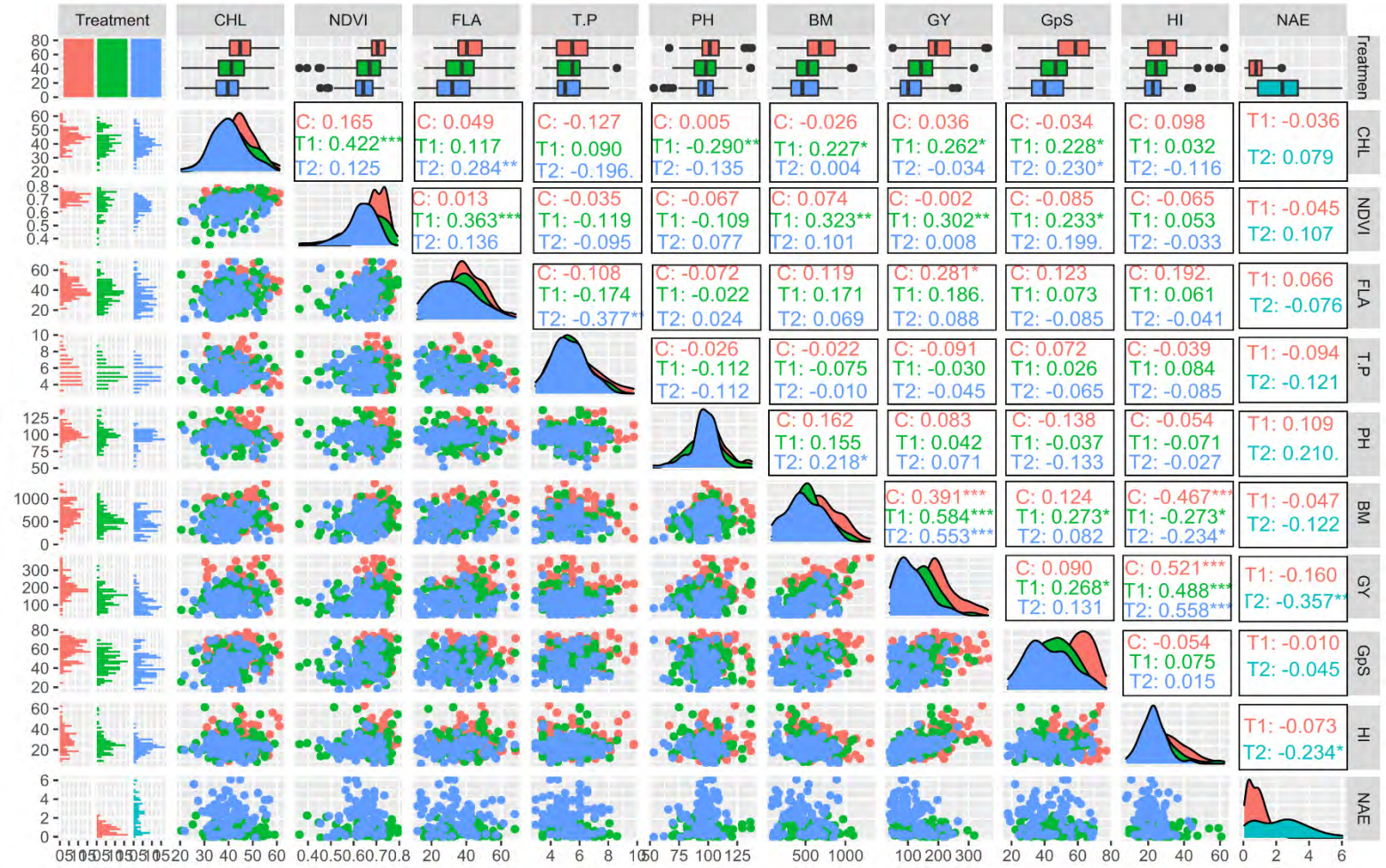

Figure 2.1. Correlation between agro-physiological traits in form of Scatterplots, histograms, boxplots and correlation coefficient (r) under variable nitrogen application; control (C; red color), treatment 1 (T1; green color) and treatment 2 (T2; Blue color). *Chlorophyll Content (CHL), Normalized Difference Vegetative Index (NDVI), Tillers Per Plant (T.P), Plant Height (PH), Flag Leaf Area (FA), Biomass(BM), Grain Yield (GY), Grains Per Spike (GpS), Harvest Index (HI), Nitrogen Agronomic Efficiency (NAE).

2.4.2. Population structure and linkage disequilibrium

Population structure was determined using the rate of change in log probability between K values. The graph of K against ΔK showed a break in the slops at K= 7 which indicated that cultivars were divided into seven sub-groups. Group-1 consisted of post green revolution cultivars adapted to irrigated areas, Group-2 included post green revolution cultivars adapted to rain fed areas, Group-3 included landraces and their derivatives, Group-4 comprised of green revolution cultivars and their derivatives, Group-5 included green revolution cultivars adapted from CIMMYT, Group-6 included post green revolution cultivars adapted from CIMMYT and Group-7 composed of elite cultivars having Inqalab-91 genetic background. The population structure revealed that about 65% of the cultivars had admixture and 35% of the population had single genetic background (Figure 2.2). LD was estimated in TASSEL standalone 5.0 for each of the three wheat sub-genomes (A, B & D). The distance at which LD decayed to half of its maximum value (r^2 value) was considered as LD decay distance. This was 300, 800 and 500 Kb for A, B and D sub-genomes, respectively.

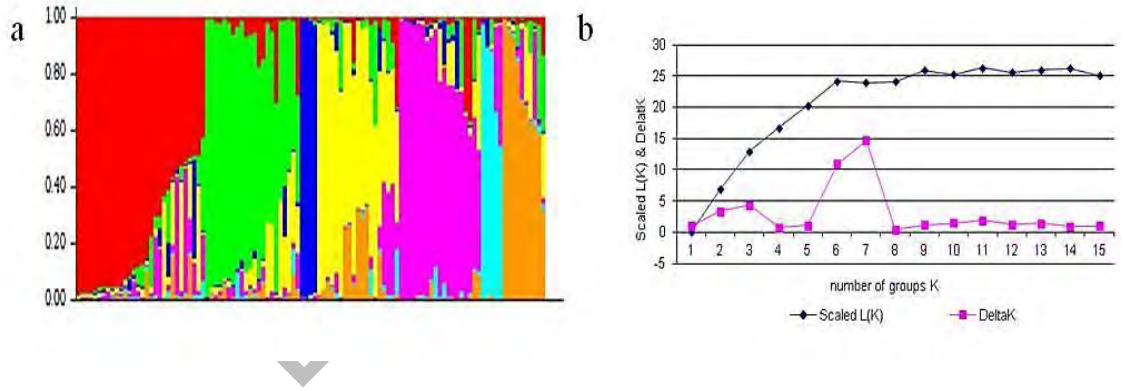

Figure 2.2. Population structure of the mapping panel. (a) The average logarithm of probability of likelihood and delta K, where K =7, (b) Membership co-efficient showing whole population is partitioned into seven sub-populations.

2.4.3. Marker trait association analysis

rMVP detected a total of 1412 MTAs [FarmCPU (560), GLM (638), and MLM(214)] associated with CHL, NDVI, FLA, PH, TP, BM, GY, GpS, HI, and NAE at -log10(p)>3 under three variable N application rate (Appendix 2.2). Of total 1412 MTAs, highest number (189) of MTAs were present on 1B chromosome and lowest number were present on 4D and 6D chromosome (11 MTAs on each chromosome). On sub-genome B, 628 MTA were detected followed by sub-genome A and D with 600 and 184 MTAs respectively. All agro-physiological

traits were found to be associated different markers in form of MTAs. Flag leaf area under treatment 1 (FLA_T1) is associated with 185 markers thus showing maximum number of marker trait association followed by chlorophyll content under control (CHL_C) with 138 MTAs. While, chlorophyll content under treatment 2 (CHL_T2) and nitrogen agronomic efficiency under treatment 1 showed minimum marker trait association i.e. 9 MTA for each trait. Harvest index under treatment 1 (HI_T1) could be a potential trait for further validation with -log10(p)=10.64. It is found to be associated with marker BobWhite_c6759_365 on chromosome 5A and detected by FarmCPU method (Appendix 2.2).

A total of 540 MTAs [FarmCPU (272), GLM (200), and MLM (68)] were found to be associated with all ten agronomic traits at -log10(p)>3 under control (N= 120 kg/ha, Appendix 2.2). Under control condition in our experiment, on sub-genome B, 277 MTA were detected followed by sub-genome A and D with 194 and 69 MTAs respectively. Highest number of MTAs (148) on 1B with lowest number (2 MTAs) on 5D chromosome. CHL_C is associated with 138 markers thus having maximum MTAs while, HI_C is associated with 28 markers thus showing minimum marker trait association under maximum application of N fertilizer (n=120 kg/ha) in field trials. Flag leaf area is the most significant trait to be exploited under maximum N application rate having -log10(p)=5.79. It is found to be associated with marker Tdurum_contig10729_64 on chromosome 6D at position (470317575 cM) and detected by FarmCPU method (Appendix 2.2).

Under treatment 1 (N= 79.2 kg/ha), a total of 479 MTAs [FarmCPU (141), GLM (252), and MLM (86)] were found to be associated with all ten agronomic traits at -log10(p)>3 (Appendix 2.2). Under control condition in our experiment, on sub-genome A, 220 MTA were detected followed by sub-genome B and D with 202 and 53 MTAs respectively. Highest number of MTAs (94) on 1A with lowest number (1 MTA) on 4D chromosome. FLA_T1 is associated with 185 markers thus showing maximum number of marker trait associations while, NAE_T1 is associated with 9 markers thus showing minimum marker trait association under moderate application of N fertilizer (N=79.2 kg/ha) in field trials. Harvest index (HI_T1) is the most significant trait to be exploited under moderate N application rate having -log10(p)=10.64. It is found to be associated with marker BobWhite_c6759_365 on chromosome 5A at position (488262509 cM) and detected by FarmCPU method.

Under treatment 2 (N= 39.6 kg/ha), a total of 393 MTAs [FarmCPU (147), GLM (186), and MLM (60)] were found to be associated with all ten agronomic traits at -log10(p)>3

(Appendix 2.2)., on sub-genome A, 186 MTA were detected on sub-genome A followed by sub-genome B and D with 145 and 62 MTAs respectively under treatment 2 (T2) in our experiment. Highest number of MTAs (55) on 6A with lowest number (2 MTA) on 6D chromosome. PH_T2 is associated with 89 markers thus showing maximum number of marker trait associations while, CHL_T2 is associated with 9 markers thus showing minimum marker trait association under minimum application of N fertilizer (N=39.6 kg/ha) in field trials. Grain yield (GY_T2) is the most significant trait to be exploited under minimum N application rate having -log10(p)=6.44. It is found to be associated with marker wsnp_Ex_c472_935980 on chromosome 5A at position (568269292 cM) and detected by FarmCPU method (Appendix 2.2).

Out of total 1412 MTAs, 274 were statistically significant with -log10(p) ≥3.7 (the threshold calculated via Bonferroni correction). Out of 274 statistically significant MTAs, 72 were detected by more than one method and scattered over different loci including all 21 chromosomes pertaining to all three wheat sub-genomes on the basis of LD decay distance (Table 2.2).

Table 2.2 . Identification of target loci based on the significant SNPs from the GWAS results.

Locus	Locus ID	Chr	Tag SNP	Tag SNP Pos	Tag SNP LOG10(p)	Method	Trait
1	q1A-1	1A	BS00081002_51	535434824	4.45	FarmCPU,GLM,MLM	CHL_T2
2	q1A-2	1A	Kukri_c44895_88	564749691	4.09	FarmCPU,GLM	PH_C
3	q1A-3	1A	Tdurum_contig5560_193	593287138	4.17	FarmCPU,GLM	NDVI_C
4	q1B-1	1B	BS00022551_51	583446285	5.37	FarmCPU,GLM,MLM	GY_C,NAE_T1
5	q1B-2	1B	CAP7_rep_c6866_212	172383664	4.07	FarmCPU,GLM	GY_C
6	q1B-3	1B	Excalibur_c60931_1260	563030480	4.01	FarmCPU,GLM	HI_C
7	q1B4	1B	Excalibur_rep_c101787_89	608996477	4.64	FarmCPU,GLM,MLM	CHL_T1
8	q1B-5	1B	JD_c107_683	563675996	5.25	FarmCPU,GLM,MLM	GY_C
9	q1B-6	1B	Ku_c1932_1583	584156264	4.4	FarmCPU,GLM	GY_C
10	q1B-7	1B	Kukri_c8143_355	581201878	3.8	FarmCPU,GLM	GY_C
11	q1B-8	1B	Kukri_c8235_371	560494382	4.31	FarmCPU,GLM	BM_C
12	q1D-1	1D	GENE-0487_795	426416291	4.35	FarmCPU,GLM	GY_C
13	q1D-2	1D	RAC875_rep_c69721_835	101942866	4.83	FarmCPU,GLM,MLM	NAE_T2
14	q2A-1	2A	CAP7_c2791_231	551720266	3.8	FarmCPU,GLM	T/P_C
15	q2A-2	2A	Tdurum_contig50824_58	550536090	3.8	FarmCPU,GLM	T/P_C
16	q2B-1	2B	Excalibur_c10071_213	692461127	4.09	FarmCPU,GLM	CHL_T1
17	q2B-2	2B	BS00046165_51	697510334	4.18	FarmCPU,GLM	NDVI_C
18	q2B-3	2B	Excalibur_c47745_63	704721642	4.05	FarmCPU,GLM	PH_T2
19	q2B-4	2B	IAAV6032	786229451	3.99	FarmCPU,GLM	BM_C
20	q2B-5	2B	RAC875_c63112_460	239646009	4.35	FarmCPU,GLM	NDVI_T2
21	q2B-6	2B	Tdurum_contig12589_325	516531745	3.8	FarmCPU,MLM	T/P_C

22	q2B-7	2B	Tdurum_contig20589_247	238961085	4.35	FarmCPU,GLM	NDVI_T2
23	q2B-8	2B	Tdurum_contig30210_226	28415893	4.66	FarmCPU,GLM,MLM	T/P_C
24	q2D-1	2D	BS00036456_51	592788886	4.73	FarmCPU,GLM	PH_C, PH_T2
25	q3A-1	3A	Excalibur_c11079_749	32201535	5.65	FarmCPU,GLM,MLM	PH_T2
26	q3A-2	3A	Kukri_c49280_230	20134735	5.46	FarmCPU,GLM	GY_T2
27	q3A-3	3A	Ra_c5515_2396	514111849	4.19	FarmCPU,GLM	PH_C
28	q3A-4	3A	Tdurum_contig5009_735	741240361	4.24	GLM,MLM	HI_T1
29	q3B-1	3B	RAC875_c55214_932	463255491	4.51	FarmCPU,GLM,MLM	NDVI_C
30	q3B-2	3B	JD_c23336_253	9170025	5.08	FarmCPU,GLM,MLM	HI_T1
31	q3B-3	3B	Kukri_rep_c83522_342	820286771	4.91	GLM,MLM	HI_T1
32	q3B-4	3B	RFL_Contig3626_521	1617465	4.04	FarmCPU,GLM	GY_T1
33	q3D-1	3D	BobWhite_c621_1218	32204706	5.65	FarmCPU,GLM,MLM	PH_T2
34	q3D-2	3D	Excalibur_c25515_95	28331150	3.93	FarmCPU,GLM	PH_T1
35	q3D-3	3D	JD_c42309_341	607001306	4.91	GLM,MLM	HI_T1
36	q3D-4	3D	Kukri_c4230_398	606862789	4.91	GLM,MLM	HI_T1
37	q3D-5	3D	Ra_c23432_639	559184550	4.2	FarmCPU,GLM	T/P_C
38	q3D-6	3D	Ra_c6639_1170	606880474	4.91	GLM,MLM	HI_T1
39	q3D-7	3D	wsnp_Ex_rep_c66380_64574083	606883054	4.85	FarmCPU,GLM,MLM	HI_T1
40	q4A-1	4A	RAC875_c59673_188	681669073	5.1	GLM,MLM	HI_T1
41	q4A-2	4A	RAC875_c59673_500	681670845	5.1	GLM,MLM	HI_T1
42	q4A-3	4A	RAC875_c7978_362	48620433	5.72	GLM,MLM	GY_T2
43	q4B-1	4B	CAP11_rep_c4893_84	10437558	4.35	GLM,NLM	GY_T2
44	q4B-2	4B	wsnp_Ex_rep_c67159_65649966	637390195	3.81	FarmCPU,GLM	BM_C
45	q4D-1	4D	BobWhite_c6759_365	488262509	10.6	FarmCPU,GLM,MLM	HI_T1
46	q4D-2	4D	BS00022191_51	476402782	3.81	FarmCPU,GLM	T/P_T1,T/P_T2
47	q4D-3	4D	Excalibur_c112658_300	457521085	3.85	FarmCPU,GLM	GY_C
48	q4D-4	4D	RAC875_c1219_1258	476603826	3.79	FarmCPU,GLM	T/P_T1,T/P_T2
49	q4D-5	4D	wsnp_Ex_c11573_18650189	482372063	3.73	FarmCPU,GLM	BM_C
50	q4D-6	4D	wsnp_Ex_c19647_28632894	470033346	4.01	FarmCPU,GLM	T/P_T1
51	q5B-1	5B	BobWhite_c15585_87	68846580	4.05	FarmCPU,GLM	FLA_T1
52	q5B-2	5B	BS00067028_51	70441099	4.23	FarmCPU,GLM	FLA_T1
53	q5B-3	5B	BS00074315_51	61381215	4.23	FarmCPU,GLM	FLA_T1
54	q5B-4	5B	Excalibur_c5540_1197	68359590	3.72	FarmCPU,GLM	FLA_T1
55	q5B-5	5B	GENE-0782_747	56565862	4.16	FarmCPU,GLM	FLA_T1
56	q5B-6	5B	IAAV4252	65243708	3.72	FarmCPU,GLM	FLA_T1
57	q5B-7	5B	IACX9238	587127034	5.36	FarmCPU,GLM,MLM	GY_T2
58	q5B-8	5B	JD_c16284_736	63362199	4.23	FarmCPU,GLM	FLA_T1
59	q5B-9	5B	Kukri_c439_857	64736979	3.72	FarmCPU,GLM	FLA_T1
60	q5B-10	5B	RAC875_c2440_755	64732501	3.72	FarmCPU,GLM	FLA_T1
61	q5B-11	5B	wsnp_Ex_c2904_5355509	60794454	4.23	FarmCPU,GLM	FLA_T1
62	q5D-1	5D	RAC875_c5518_1401	74464487	4.23	FarmCPU,GLM	FLA_T1
63	q6A-1	6A	BobWhite_c1082_134	548411545	3.81	FarmCPU,GLM	NAE_T2
64	q6A-2	6A	IAAV4703	549036170	4.3	FarmCPU,GLM	NAE_T2
65	q6A-3	6A	Tdurum_contig42125_5972	545828799	4.82	FarmCPU,GLM,MLM	NAE_T2
66	q6B-1	6B	Kukri_c75359_152	681317076	4.61	FarmCPU,GLM,MLM	CHL_C
67	q6B-2	6B	RAC875_c5413_1237	710006969	4.42	FarmCPU,GLM	NDVI_T1

68	q6D-1	6D	Tdurum_contig10729_64	470317575	5.79	FarmCPU,GLM,MLM	FLA_C
69	q7B-1	7B	IAAV3313	701187837	3.96	FarmCPU,GLM	GpS_T2
70	q7B-2	7B	Ra_c26852_957	700830514	3.99	FarmCPU,GLM	PH_T2
71	q7B-3	7B	Tdurum_contig43954_1287	701187687	3.96	FarmCPU,GLM	GpS_T2
72	q7D-1	7D	Ra_c9123_3192	9307439	4.02	FarmCPU,GLM	FLA_T1

Abbreviations: Control (C), Treatment 1 (1), Treatment (T2), Chlorophyll content (CHL), normalized difference vegetation index (NDVI), flag leaf area (FLA), tiller per plant (T.P), plant height (PH), biomass (BM), grain yield (GY), grain per spike (GpS), harvest index (HI) and nitrogen agronomic efficiency (NAE).

The rMVP presented the MTAs in form of the density plots, QQ (Quantile-Quantile) - plots and manhattan plots. Density plot shows the distribution of each trait under study. QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU for desired traits. Manhattan plot is showing the *P* values of the entire GLM, MLM, and FarmCPU analysis for traits under study. All these plots were estimated for all ten agro-physiological traits under three nitrogen treatments i.e. C, T1 and T2. The density, QQ and Manhattan plots for all traits verified by all three methods (FarmCPU, GLM and MLM) simultaneously including CHL_C, CHL_T1, CHL_T2, NDVI_T2, FLA_C, TP_C, PH_T2, GY_C, GY_T2, HI_T1, NAE_T1 and NAE_T2 (Figure 2.3- 2.14) were included in main chapters while rest of them were present in supplementary data (Appendix 2.3-2.19).

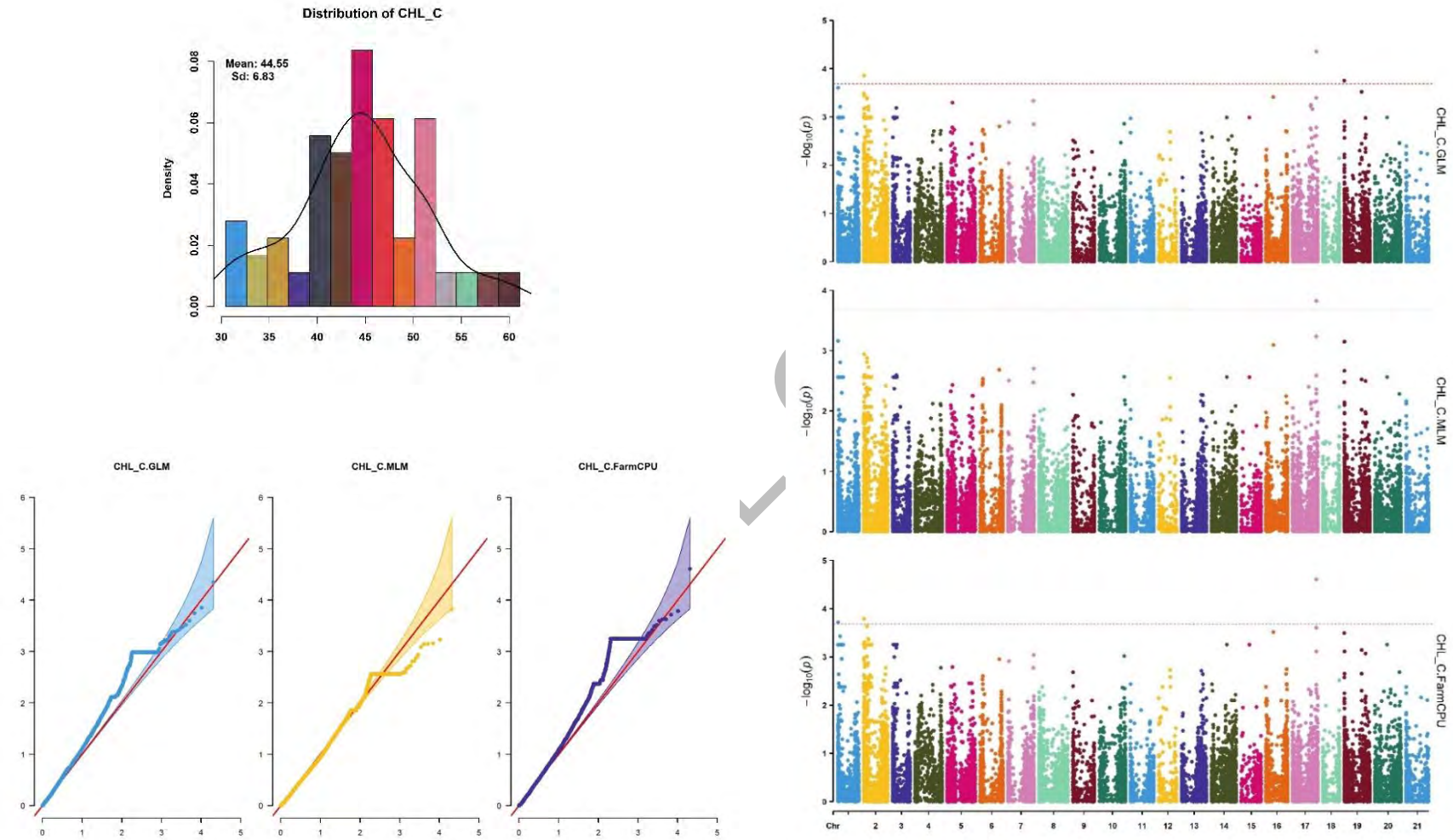

Figure 2.3. The Density Distribution plot, QQ-plot, and Manhattan plot for chlorophyll under control; CHL_C. (a) The density plot is showing the distribution of CHL_C in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected value in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.

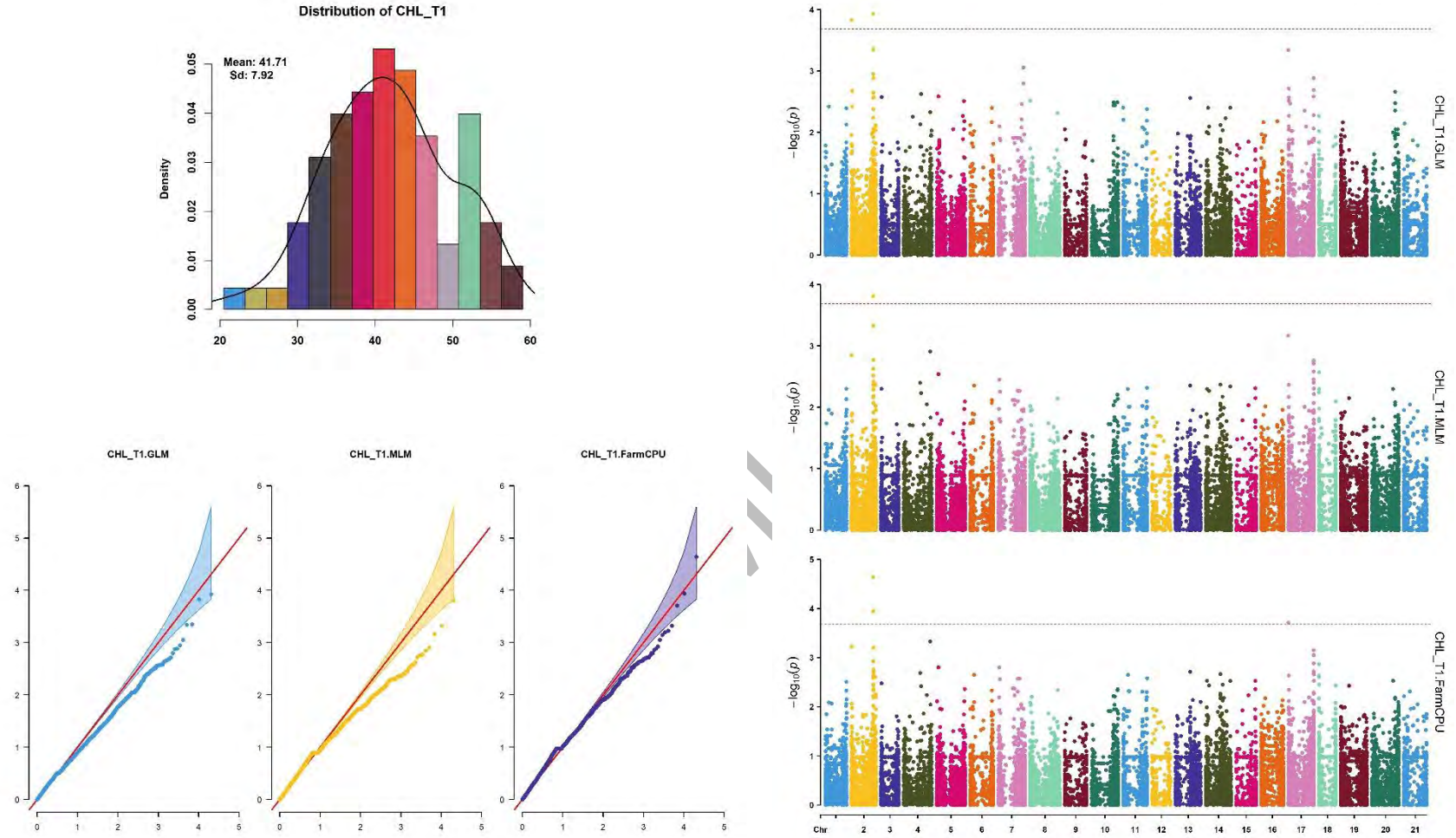

Figure 2.4. The Density Distribution plot, QQ-plot, and Manhattan plot for chlorophyll under treatment 1; CHL_T1. (a) The density plot is showing the distribution of CHL_T1 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.

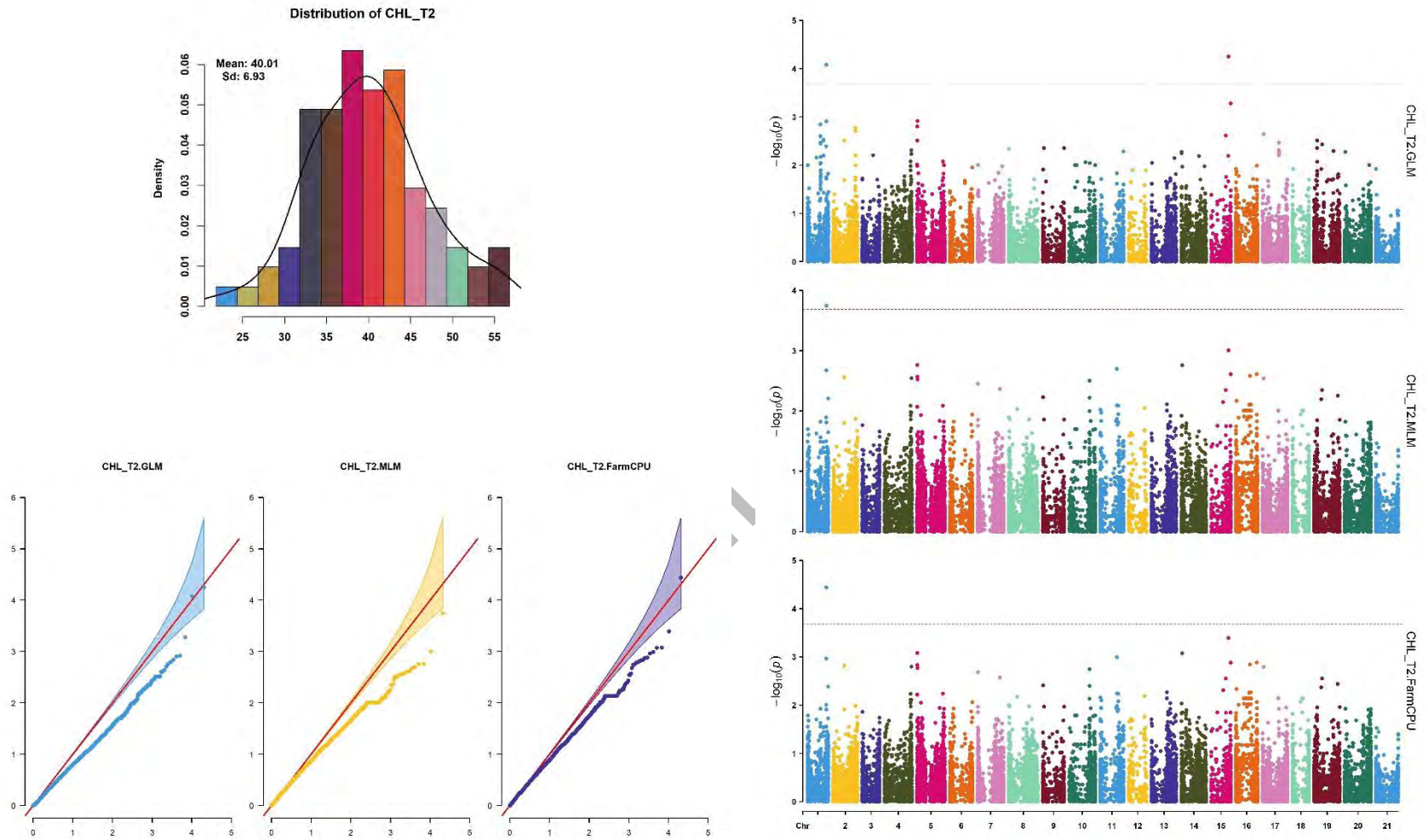

Figure 2.5. The Density Distribution plot, QQ-plot, and Manhattan plot for chlorophyll under treatment 2; CHL_T2. (a) The density plot is showing the distribution of CHL_T2 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.

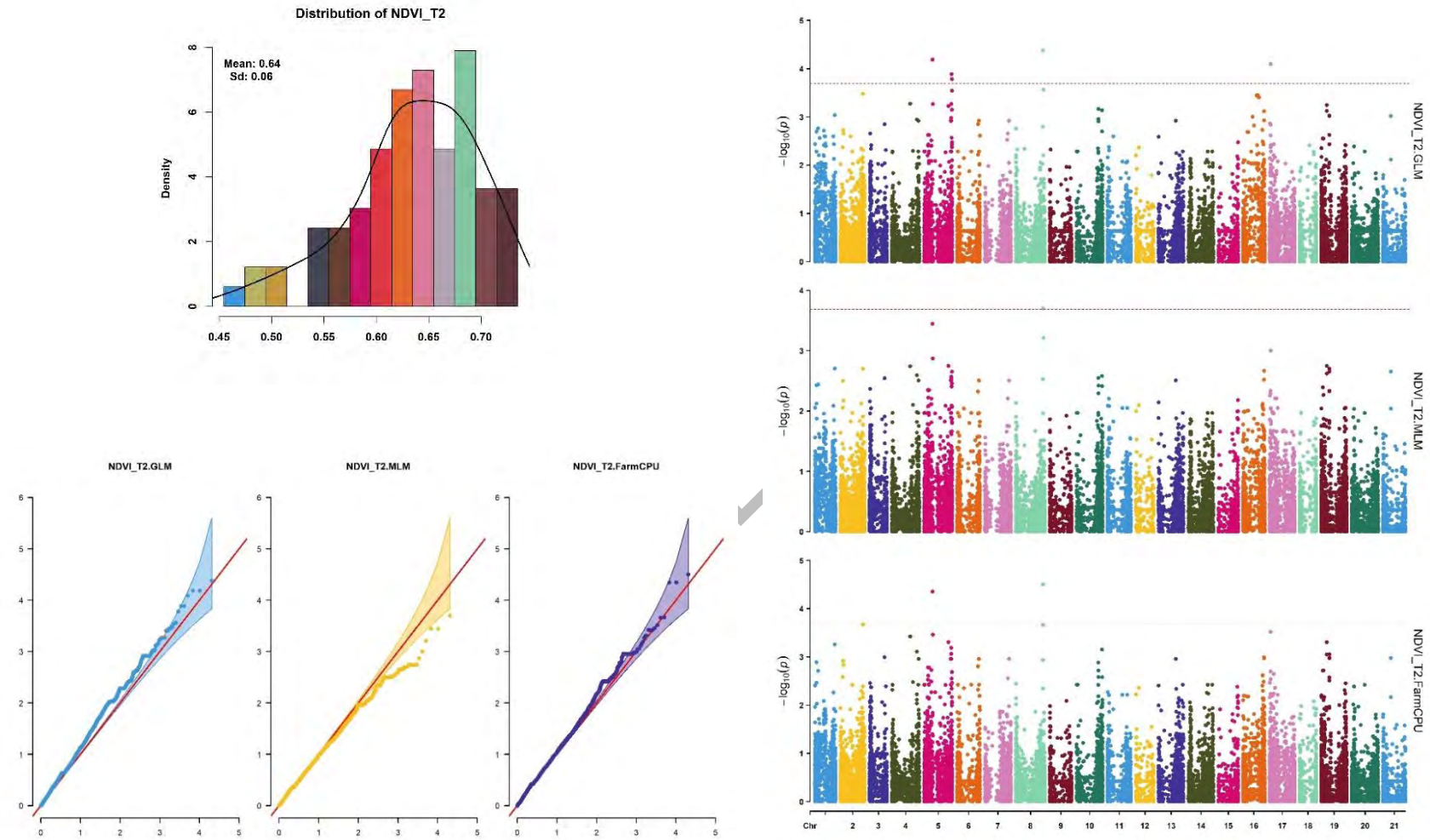

Figure 2.6. The Density Distribution plot, QQ-plot, and Manhattan plot for NDVI under treatment 2; NDVI_T2. (a) The density plot is showing the distribution of NDVI_T2 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.

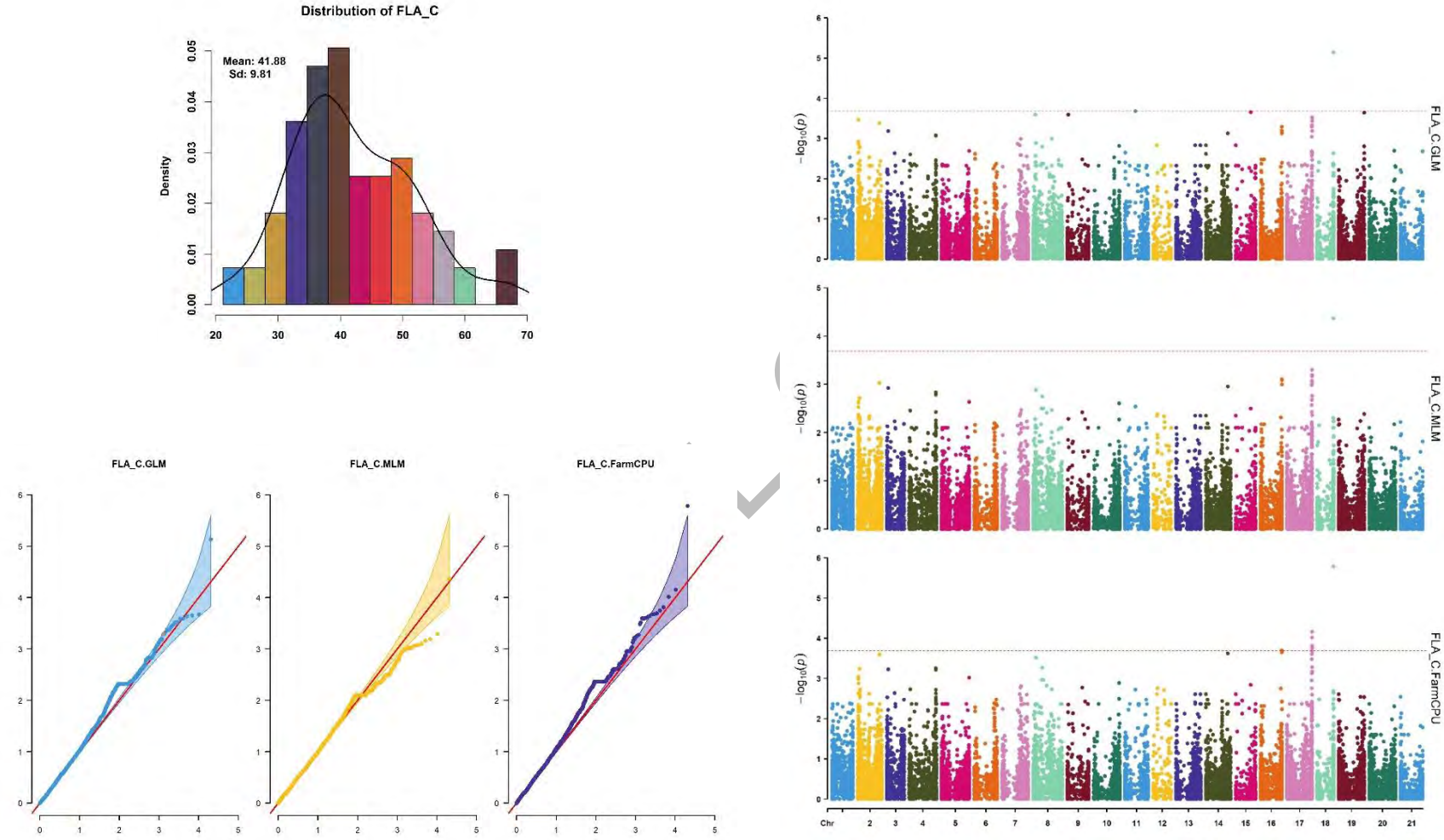

Figure 2.7. The Density Distribution plot, QQ-plot, and Manhattan plot for flag leaf area under control; FLA_C. (a) The density plot is showing the distribution of FLA_C in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.

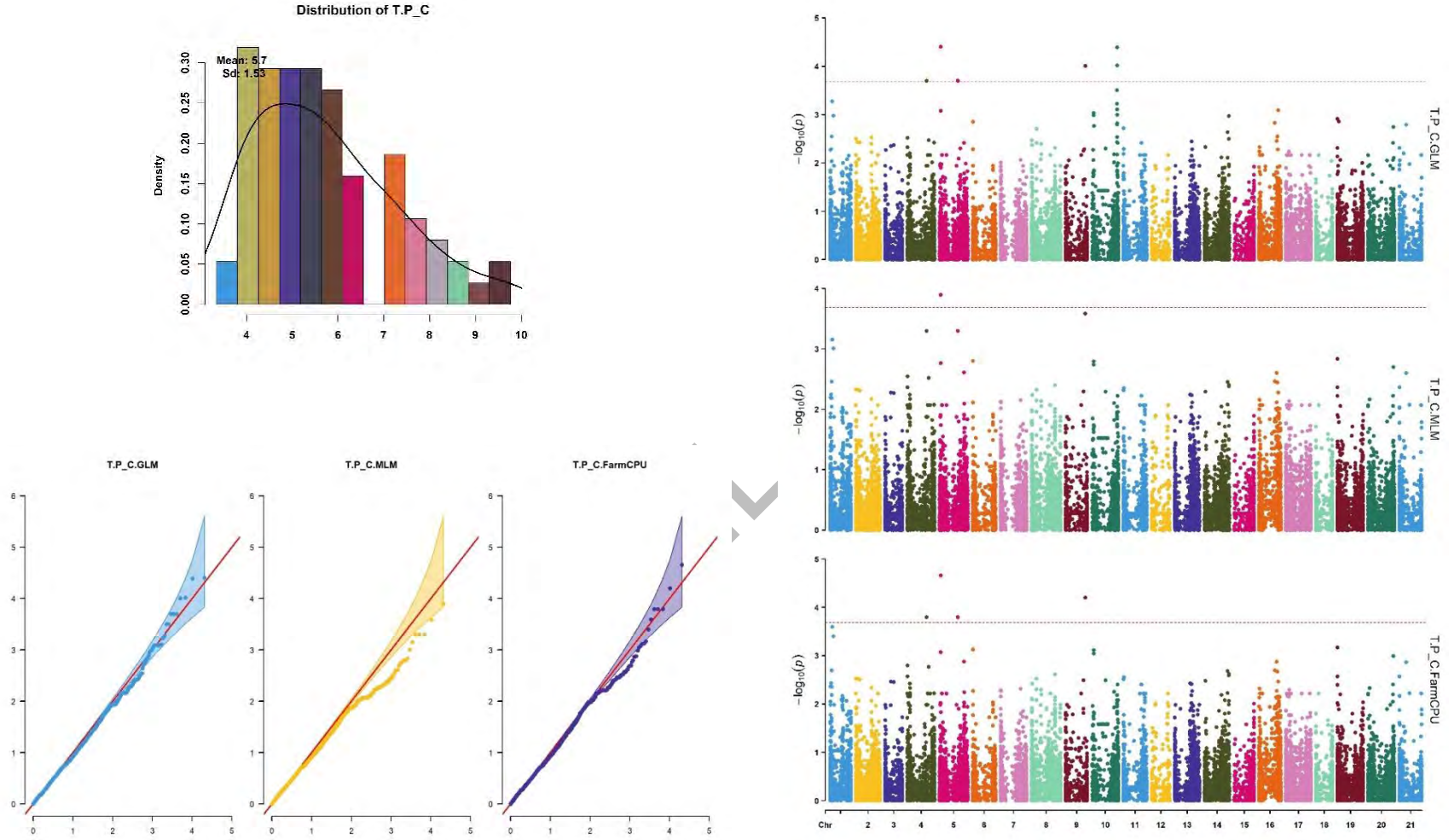

Figure 2.8. The Density Distribution plot, QQ-plot, and Manhattan plot for tiller per plant under control; T.P_C. (a) The density plot is showing the distribution of T.P_C in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.

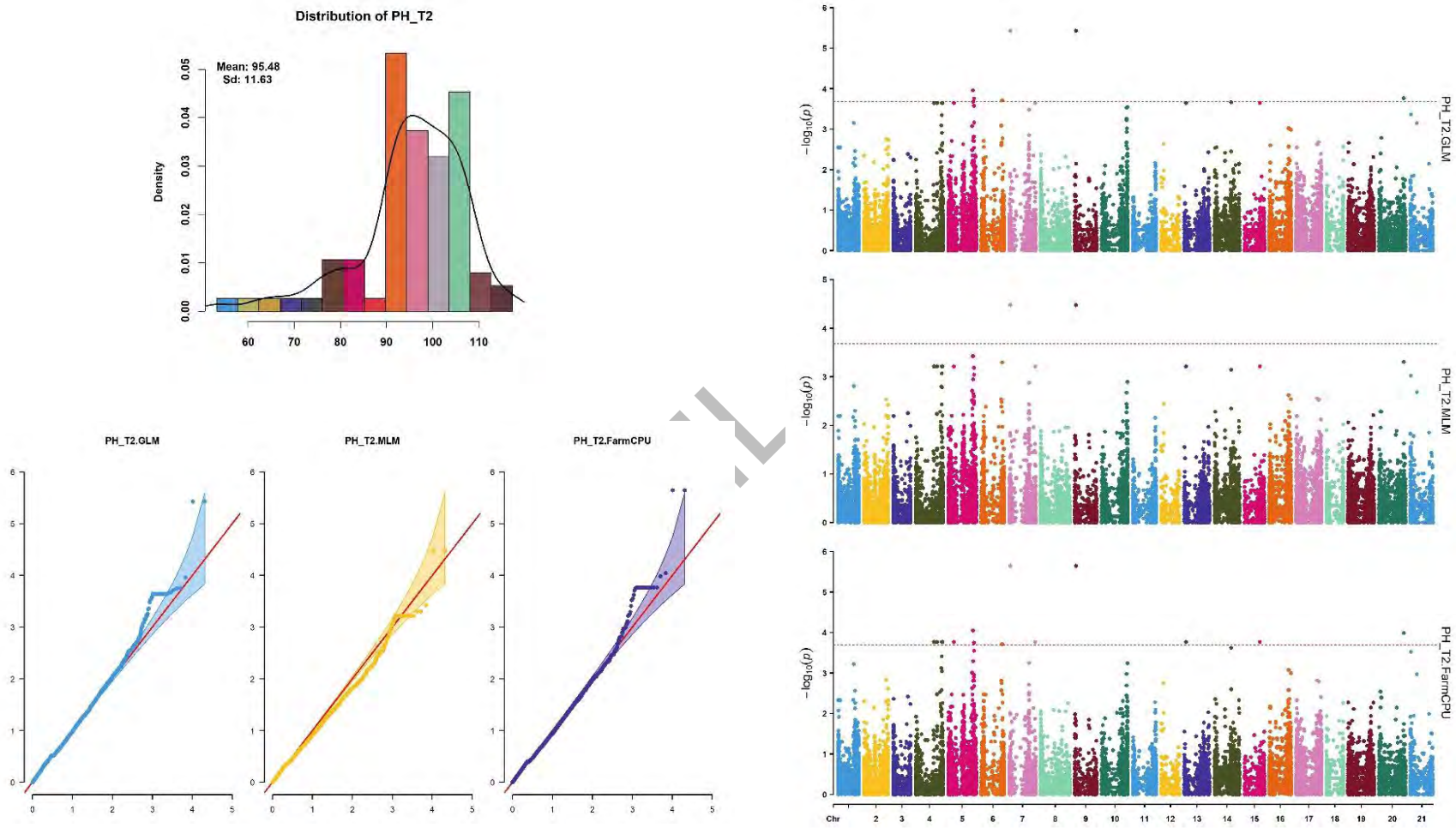

Figure 2.9. The Density Distribution plot, QQ-plot, and Manhattan plot for plant height under treatment 2; PH_T2. (a) The density plot is showing the distribution of PH_T2 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.

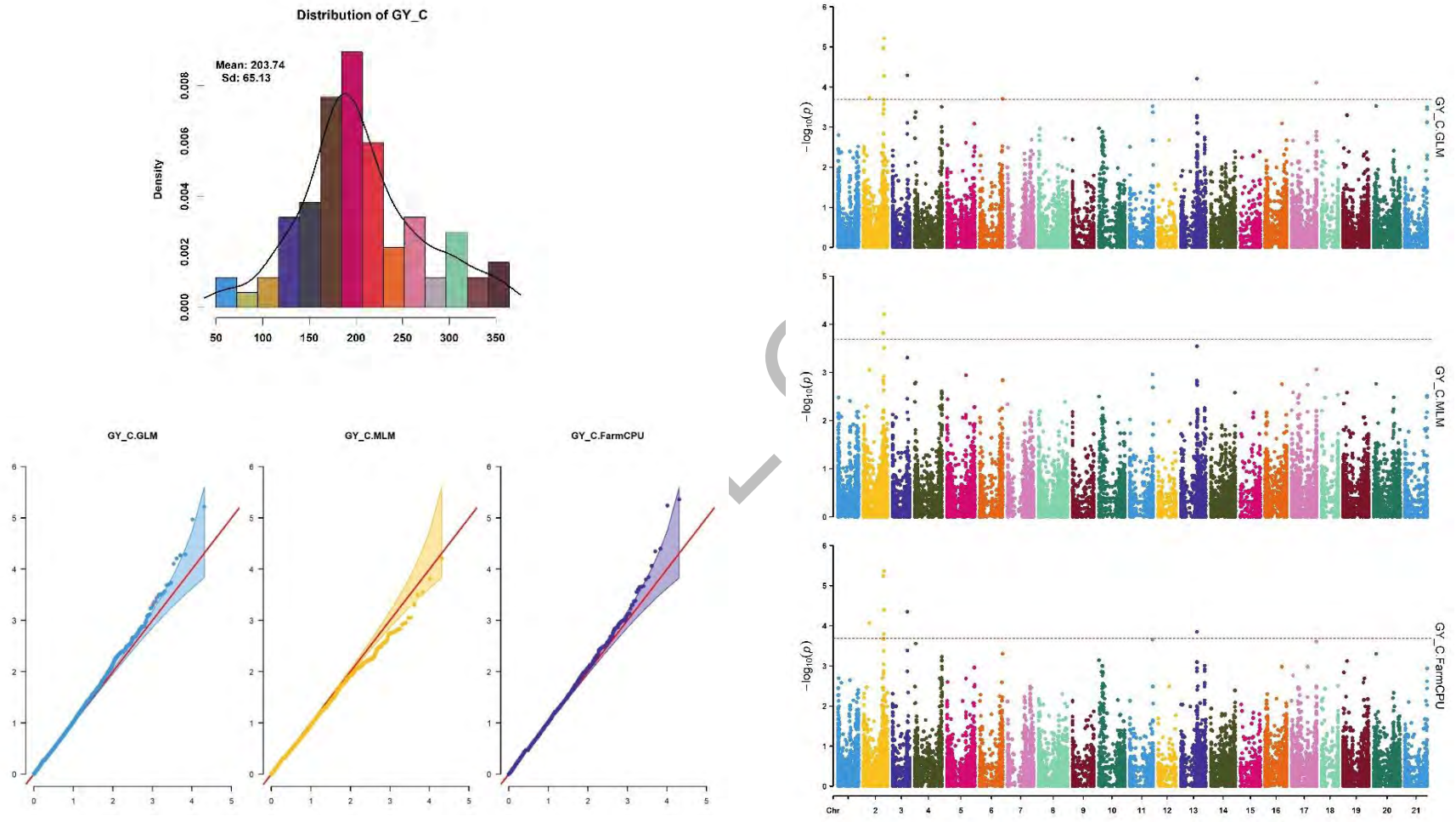

Figure 2.10. The Density Distribution plot, QQ-plot, and Manhattan plot for grain yield under control; GY_C. (a) The density plot is showing the distribution of GY_C in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.

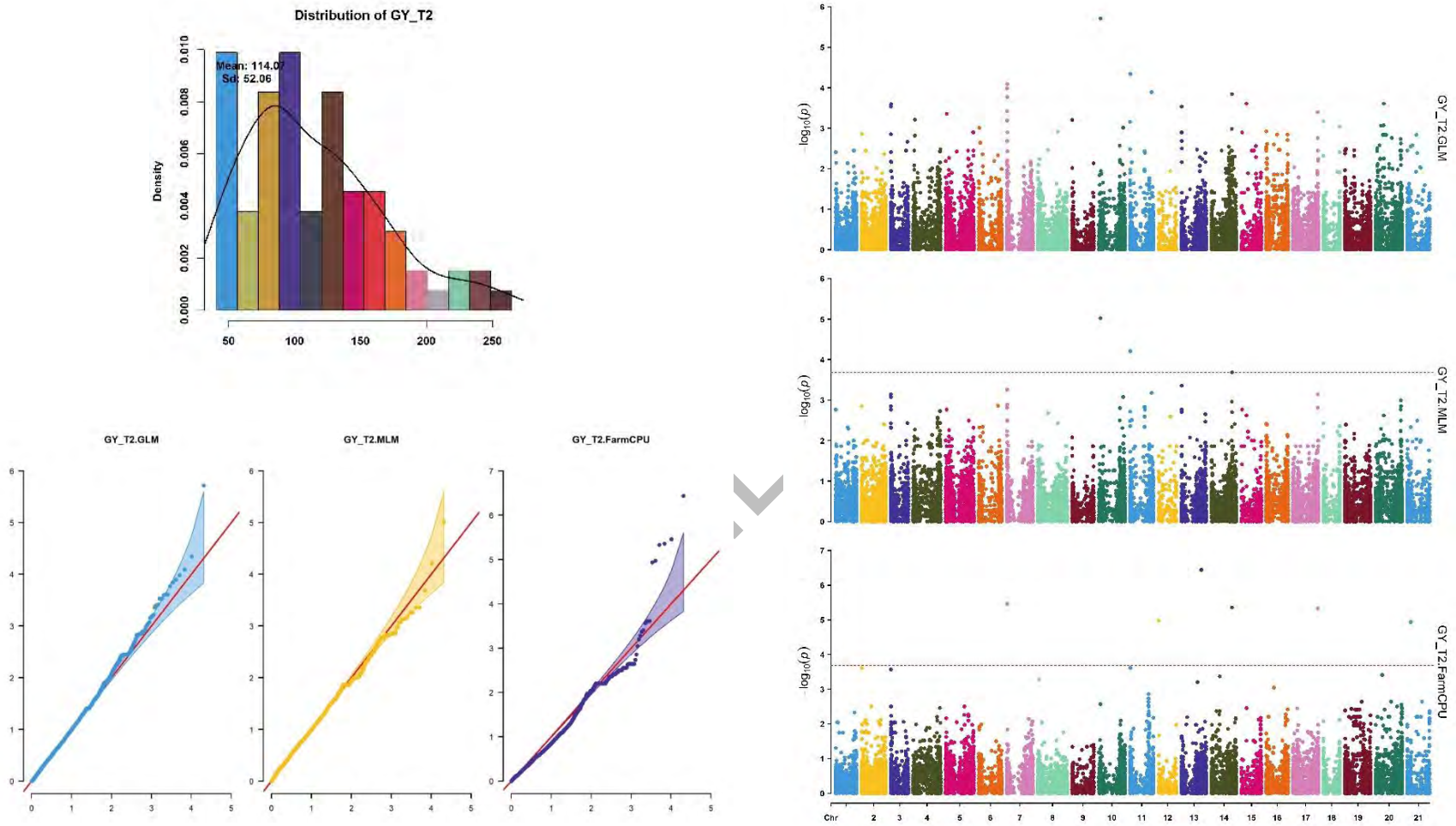

Figure 2.11 The Density Distribution plot, QQ-plot, and Manhattan plot for grain yield under treatment 2; GY_T2. (a) The density plot is showing the distribution of GY_T2 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.

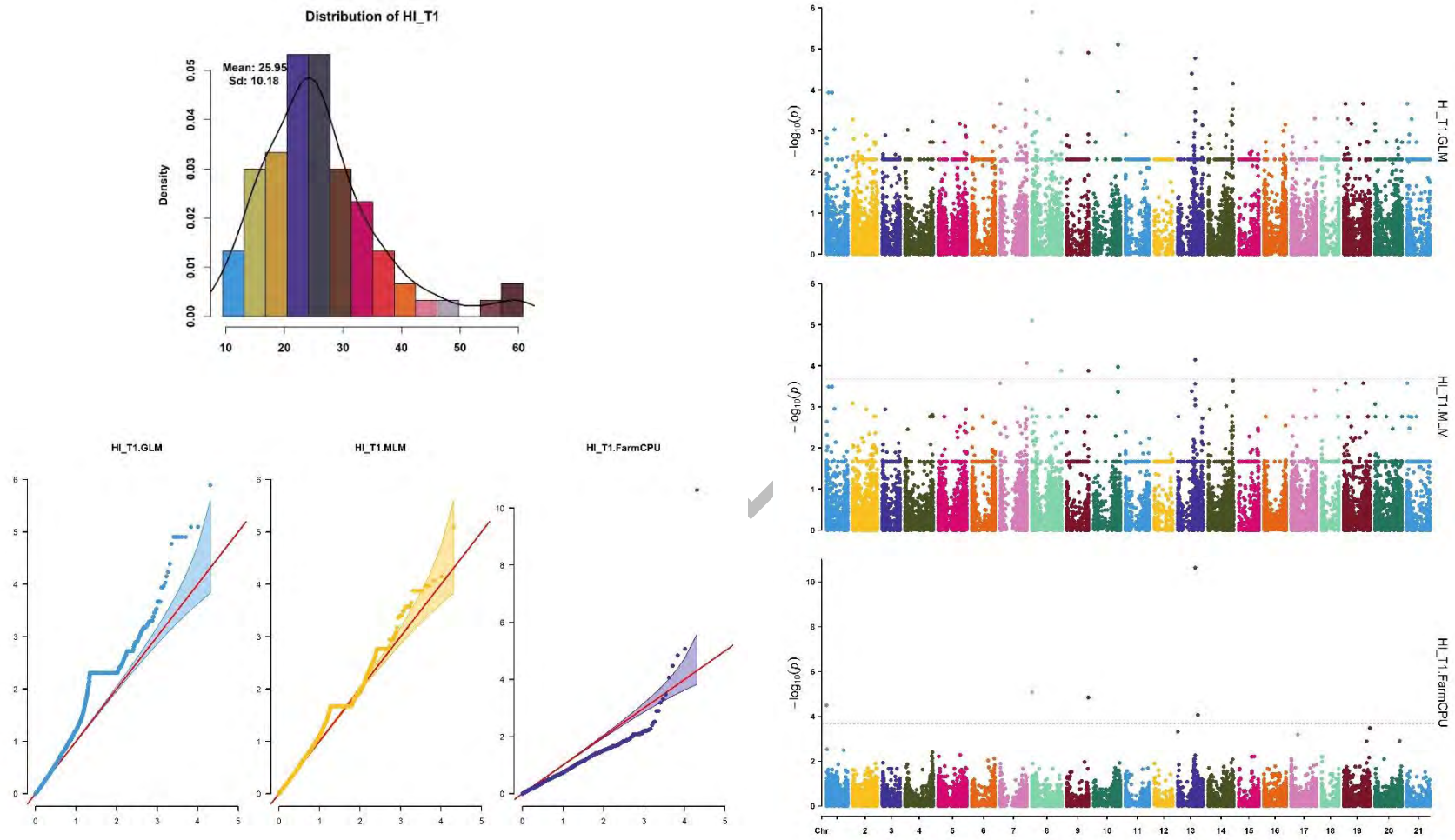

Figure 2.12. The Density Distribution plot, QQ-plot, and Manhattan plot harvest index under treatment 1; HI_T1. (a) The density plot is showing the distribution of HI_T1 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.

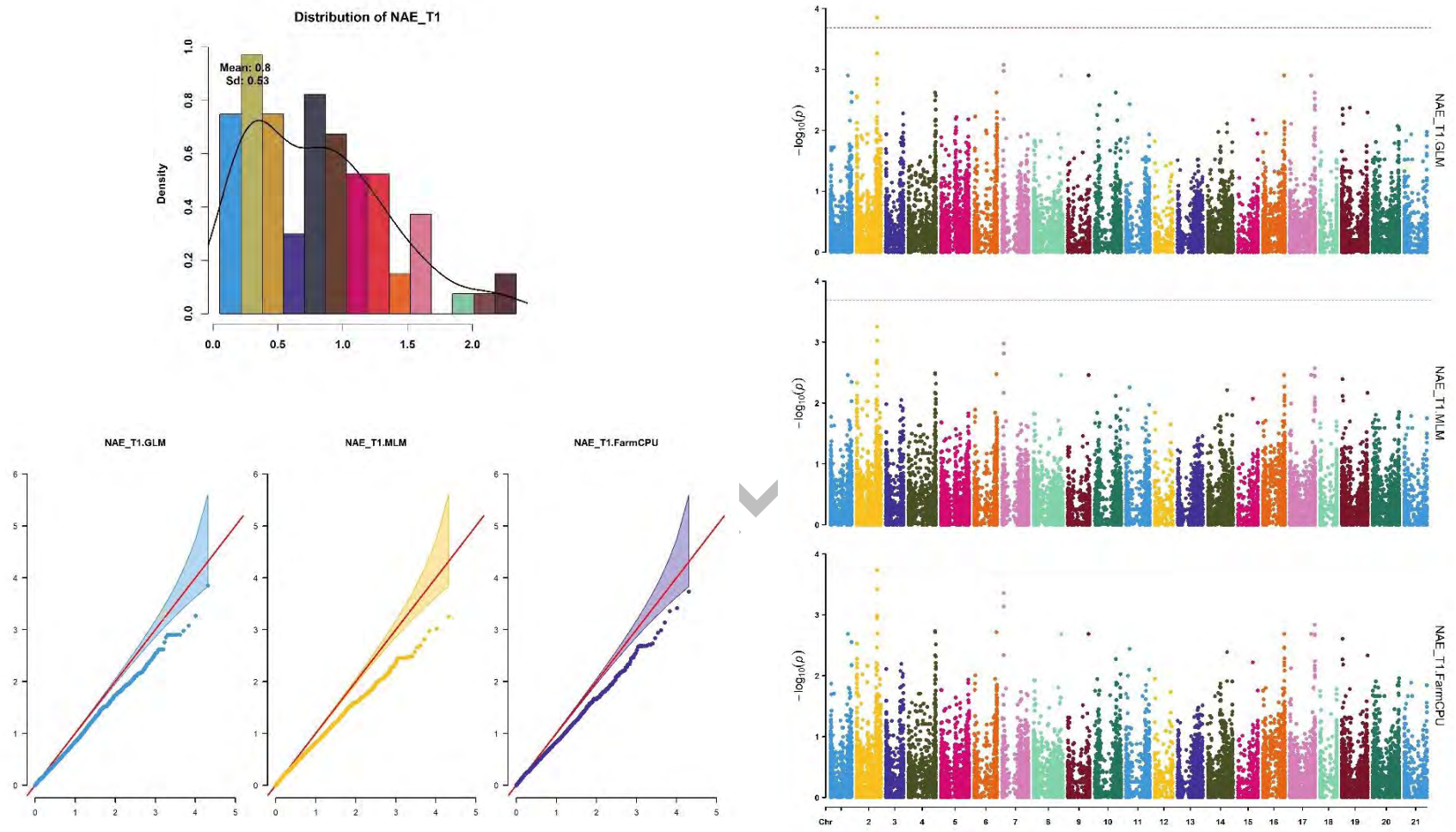

Figure 2.13.The Density Distribution plot, QQ-plot, and Manhattan plot for nitrogen agronomic efficiency under treatment 1; NAE T1. (a) The density plot is showing the distribution of NAE T1 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.

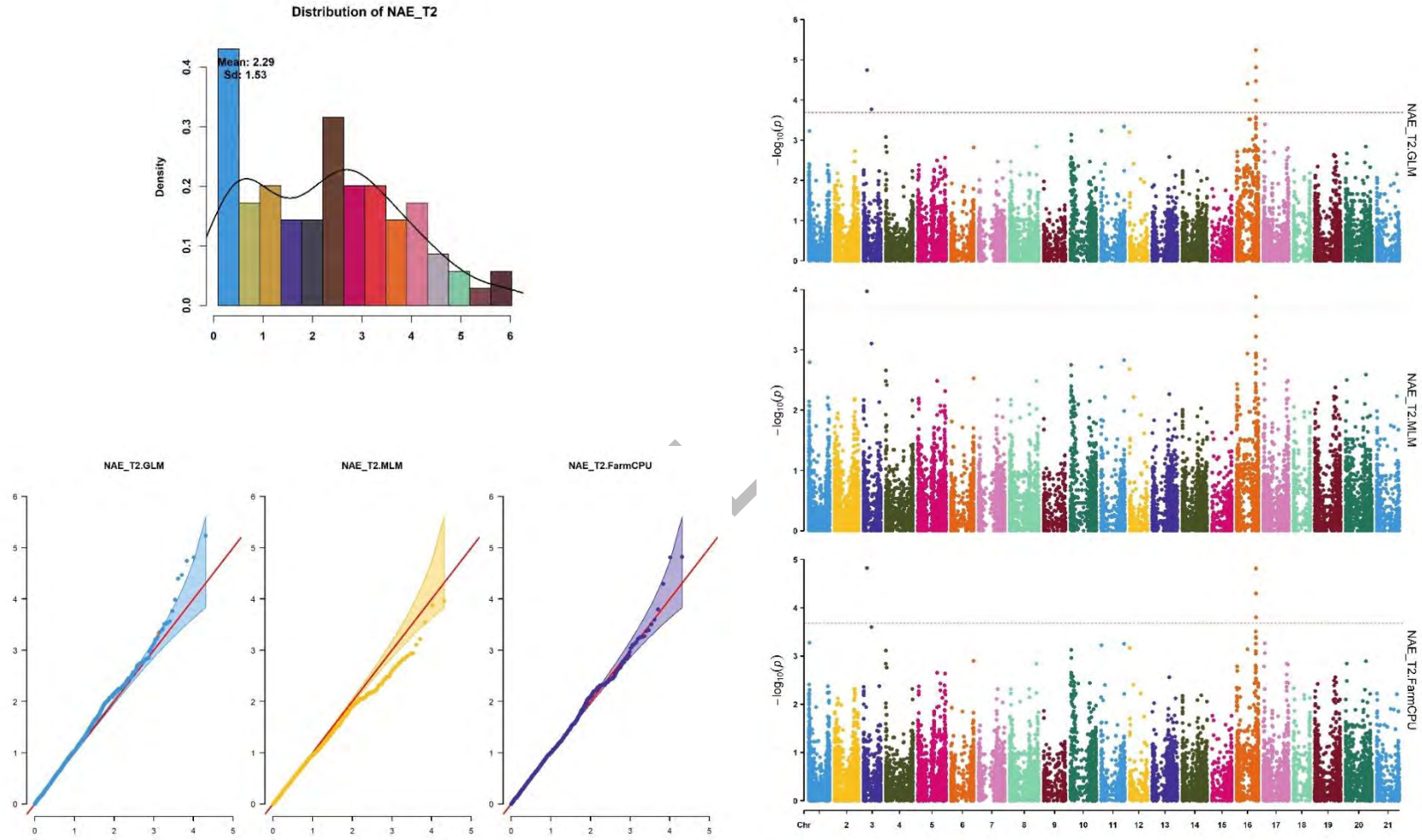

Figure 2.14. The Density Distribution plot, QQ-plot, and Manhattan plot for nitrogen agronomic efficiency under treatment 2; NAE T2. (a) The density plot is showing the distribution of NAE T2 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.

2.4.4. Genes of interest

Of the 72 loci, each identified by more than one model were used to extract genes from *Ensembl Plants* database for wheat genes using BioMart function. The known high confidence protein coding genes encode proline iminopeptidase, defense response, protein glycosylation, metal ion binding protein, bidirectional sugar transporter (SWEET), protein involved in negative regulation of transcription, annexin, integral component of membrane, protein involved in nucleus structural formation, regulation of transcription, DNA-templated, integral component of membrane, protein metabolic process, ATP and nucleic acid binding, ATP hydrolysis, mitochondrial intermembrane space, SCF complex assembly, RBR-type E3 ubiquitin transferase, probable inactive DNA (cytosine-5)-methyltransferase DRM3, UTP--glucose-1-phosphate uridylyltransferase, DNA topoisomerase type II, kinesin-like protein, protein dimerization activity, dexh-box ATP-dependent RNA helicase dexh15 chloroplastic, GTP binding protein, Pyrophosphate hydrolysis-driven proton transmembrane transporter activity (Table 2.3).

Table 2.3 . Loci identified by multiple GWAS methods selected for candidate gene analysis.

No	Trait	SNP	CHROM	POS	Method	log10(p)	Wheat gene ID	Gene description
1	GY_C	GENE-0487_795	1D	426416291	FarmCPU,GLM	4.35	TraesCS1A02G333700	Proline iminopeptidase
2	GY_C	JD_c107_683	1B	563675996	FarmCPU,GLM,MLM	5.25	TraesCS1B02G336700	Defense response
3	GY_C,NAE_T1	BS00022551_51	1B	583446285	FarmCPU,GLM,MLM	5.37	TraesCS1B02G352700	Protein glycosylation
4	GY_C	Ku_c1932_1583	1B	584156264	FarmCPU,GLM	4.4	TraesCS1B02G354400	Metal ion binding
5	CHL_T1	Excalibur_rep_c101787_89	1B	608996477	FarmCPU,GLM,MLM	4.64	TraesCS1B02G377200	Bidirectional sugar transporter SWEET
6	T/P_C	CAP7_c2791_231	2A	551720266	FarmCPU,GLM	3.8	TraesCS2A02G322400	Negative regulation of transcription
7	NDVI_T2	RAC875_c63112_460	2B	239646009	FarmCPU,GLM	4.35	TraesCS2B02G238300	Annexin
8	BM_C	IAAV6032	2B	786229451	FarmCPU,GLM	3.99	TraesCS2B02G605000	Integral component of membrane
9	HI_T1	Kukri_c4230_398	3D	606862789	GLM,MLM	4.91	TraesCS3A02G523600	Nucleus structural formation
10	HI_T1	JD_c42309_341	3D	607001306	GLM,MLM	4.91	TraesCS3A02G524800	Regulation of transcription, DNA-templated
11	NDVI_T2	BS00095515_51	3B	772397461	FarmCPU,GLM,MLM	4.51	TraesCS3B02G531400	Integral component of membrane
12	NDVI_C	BS00046164_51	2B	697510323	FarmCPU,GLM	4.09	TraesCS3D02G273600	Protein metabolic process
13	NDVI_C	BS00046165_51	2B	697510334	FarmCPU,GLM	4.18	TraesCS3D02G273600	ATP and nucleic acid binding, ATP hydrolysis
14	HI_T1	RAC875_c59673_500	4A	681670845	GLM,MLM	5.1	TraesCS4A02G408900	Annexin
15	GY_T2	CAP11_rep_c4893_84	4B	10437558	GLM,NLM	4.35	TraesCS4B02G014300	Metal ion binding
16	T/P_T1,T/P_T2	BS00022191_51	4D	476402782	FarmCPU,GLM	3.81	TraesCS5A02G263400	Mitochondrial intermembrane space
17	FLA_T1	GENE-0782_747	5B	56565862	FarmCPU,GLM	4.16	TraesCS5B02G051900	SCF complex assembly
18	FLA_T1	BS00074315_51	5B	61381215	FarmCPU,GLM	4.23	TraesCS5B02G055600	RBR-type E3 ubiquitin transferase
19	FLA_T1	JD_c16284_736	5B	63362199	FarmCPU,GLM	4.23	TraesCS5B02G057300	Probable inactive DNA (cytosine-5)-methyltransferase DRM3
20	PH_T2	Ra_c26852_957	7B	700830514	FarmCPU,GLM	3.99	TraesCS5B02G356300	UTP--glucose-1-phosphate uridylyltransferase
21	FLA_T1	IAAV4252	5B	65243708	FarmCPU,GLM	3.72	TraesCS5B02G059200	Regulation of transcription, DNA-templated
22	FLA_T1	Excalibur_c5540_1197	5B	68359590	FarmCPU,GLM	3.72	TraesCS5B02G061000	Integral component of membrane
23	FLA_T1	BS00067028_51	5B	70441099	FarmCPU,GLM	4.23	TraesCS5B02G062800	DNA topoisomerase type II
24	GY_T2	IACX9238	5B	587127034	FarmCPU,GLM,MLM	5.36	TraesCS5B02G412300	Kinesin-like protein
25	GY_C	Excalibur_c112658_300	4D	457521085	FarmCPU,GLM	3.85	TraesCS5D02G248800	Protein binding
26	HI_T1	BobWhite_c6759_365	4D	488262509	FarmCPU,GLM,MLM	10.64	TraesCS5D02G286300	Protein dimerization activity
27	NAE_T2	BobWhite_c1082_134	6A	548411545	FarmCPU,GLM	3.81	TraesCS6A02G312100	Dexh-box ATP-dependent RNA helicase dexh15 chloroplastic
28	NAE_T2	IAAV4703	6A	549036170	FarmCPU,GLM	4.3	TraesCS6A02G312300	GTP binding
29	GpS_T2	IAAV3313	7B	701187837	FarmCPU,GLM	3.96	TraesCS7B02G433800	Pyrophosphate hydrolysis-driven proton transmembrane transporter activity

Abbreviations: Control (C), Treatment 1 (1), Treatment (T2), Chlorophyll content (CHL), normalized difference vegetation index (NDVI), flag leaf area (FLA), tiller per plant (T.P), plant height (PH), biomass (BM), grain yield (GY), grain per spike (GpS), harvest index (HI) and nitrogen agronomic efficiency (NAE).

2.5. Discussion

Nitrogen occupies a distinct position as a plant nutrient as it is required in high amounts relative to the other necessary nutrients (Marschner, 1995). Effective application of nitrogen (N) is essential for attaining high quality and production in wheat. Identifying genetic basis to utilize applied N more efficiently is a potential way of reducing N losses through leaching and denitrification (Rosenstock et al., 2013). Identification of genomic polymorphism in form of SNP markers which can regulate expression of genes responsive to N levels and help plant to utilize available N efficiently can reduce the N inputs to soil which is annually lost because of leaching into waterways (Davis, 2013). Therefore the efficient use of nitrogen is needed (Asplund, 2014a). In the present study, on the basis of phenomics and genomics of Pakistani historical bread wheat panel, nitrogen responsive marker traits associations were identified with potential candidate genes involved in N pathway in wheat which can be used for future breeding programs.

As nitrogen supply has direct impact on the vigour of a crop and results in more grain yield thus N fertilization in wheat contributes to enhanced yield as observed in present work which was previously reported by other studies (Benzian & Lane, 1981; Hastenpflug et al., 2011; Mandic et al., 2015; Mansour et al., 2017; Orloff et al., 2012). Results depicted significant variations between N levels and varieties for grain yield, biomass, chlorophyll content and NDVI along with other yield components. In the present research work, phenotyping was done by using precision agricultural approaches as N response related factors specifically NDVI and CHL (chlorophyll content) were determined which directly affects NUE, NNI and grain yield. Genotypes having high CHL and NDVI also have high grain yield, NAE and biomass (Figure 2.1). These results are in line with previously reported findings (Mansour et al., 2017). In the present study, chlorophyll content has a linear correlation with applied N fertilizer and grain yield (Figure 2.1) and has been reported previously (Ali et al., 2017; Prost & Jeuffroy, 2007; Skudra & Ruža, 2017; Yang et al., 2018a). Same trend of linear correlation was observed between NDVI, GY, BM and NAE and this confirmed the finding of others (Arnall et al., 2006; Nguyen et al., 2016; Vian et al., 2018a). Complex traits including many agro-physiological traits are regulated by a number of metabolic networks and have a downstream effect on grain yield. GY showed significant variation for both treatments and varieties ($P>0.0001$) and these results are in line with many previous reports on impact of N fertilization on wheat (Guarda et al., 2004; Hussain et al., 2006; Maqsood et al., 2002b).

In GWAS analysis, we have identified MTAs through three models; FarmCPU, GLM and MLM using rMVP package. In our dataset, the most significant MTAs with log10(p)=10.64 associated with marker BobWhite_c6759_365 on chromosome 5A at position (488262509 cM) was detected by FarmCPU model in treatment 1 (N=79.2 kg/ha). In control and treatment 2, the most significant SNPs with marker named Tdurum_contig10729_64 [-log10(p)=5.79] and wsnp_Ex_c472_935980 [-log10(p)=6.44] were also detected through FarmCPU model. Similar claims have been made by other researchers. The FarmCPU (multi-locus model) is statistically more powerful than single locus models while requiring a lower level of over or under fitting of data (Li et al., 2021; Liu et al., 2018). One potential disadvantage of FarmCPU is that it identifies the single SNP that is the most significant at a specified genomic location rather than a peak with bulk of SNPs as do many other MLM models (Kaler et al., 2020).

The MTAs linked with agro-physiological traits including CHL_C, CHL_T1, CHL_T2, NDVI_T2, FLA_C, TP_C, PH_T2, GY_C, GY_T2, HI_T1, NAE_T1 and NAE_T2 in the present study were found to be located in the genomic region of the earlier reported genes involved in regulating UTP--glucose-1-phosphate uridyl transferase, bidirectional sugar transporter (SWEET), ATP and nucleic acid binding, ATP hydrolysis, mitochondrial intermembrane space, SCF complex assembly, RBR-type E3 ubiquitin transferase, DNA topoisomerase type II, kinesin-like protein (Dong et al., 2012; Reddy et al., 1999; Xie et al., 2021). Interestingly, the *GOGAT* (Glutamine oxoglutarate aminotransferase) gene accelerating the NUE in wheat being part of Gs/GOGAT cycle (Fontaine et al., 2009; García-Suárez et al., 2010; Laperche et al., 2007; Quraishiet al., 2011; Sun et al., 2013) was observed to be collocated with the SNP (RAC875_c55214_932) associated NDVI_C on Chromosome 3B in the present study (Table 2.2). Another gene named Ppd-B1 found to be associated with Excalibur_c10071_213 located on 2B chromosome and linked with chlorophyll content under treatment 1 (CHL_T1, Table 2.2). Ppd-D1 is the major gene responsible for late heading in wheat so plant can stay green for a long time and ultimately leads to more grain yield. It was previously reported in many QTL studies of wheat conducted in different environments under variable nitrogen applications (An et al., 2006a; Mahjourimajd, 2015; Quraishi et al., 2011; Ren et al., 2018). For further information on genes which have not been studied in wheat, it may be useful to look at rice which is now potentially used as a reference for grasses. Future gene expression experiments on these genes will help for in-depth analysis and more accurate information on candidate gene(s) for N responsive traits in wheat.

2.6. Conclusion

Wheat trait improvement can be divided into yield potential in a high input environment and adaptability in a low input environment. High inputs have been linked to increased genetic gains and yield performance. Examining traits and breeding with fewer resources and inputs, can be beneficial for selecting germplasm that maximises resource utilisation in a limited environment. The purpose of this study was to examine the roles of yield contributing traits in low and high N input environments. This work proved reliability and the power of multi-locus (ML)-GWAS models such as FarmCPU about N related traits in wheat and provided new insights into understanding of N pathway in wheat, which may facilitate breeding in wheat by using non-destructive precision agriculture approaches for efficient utilization of N in bread wheat. The identification of genomic regions associated with yield determining traits in historical bread wheat panel of Pakistan and then comparing these with the wheat reference genome helped to identify potential candidate genes involved in the nitrogen pathway in wheat. Identified putative candidate genes associated with significant MTAs, may be directly or indirectly involved with various biological processes, molecular functions and cellular component organization. These candidate genes might also play a key role in plant growth and development along with grain production.

Chapter#3

Statistical investigation to decrypt the contribution of grain yield components and root traits towards the final grain yield in wheat under high and low nitrogen application

Chapter # 3

Statistical Investigation to Decrypt the Contribution of Grain Yield Components and Root Traits towards the Final Grain Yield in Wheat under High and Low Nitrogen Application.

3.1. Abstract

To reduce the nitrogen (N) footprint on the ecosystem and the consequent economic burden as a result of over N fertilization, an increase in grain yield under optimum N Level is a key current goal. This study was conducted to examine the direct, indirect and total effect of grain yield components (GYC) and root traits (RT) on grain yield (GY) of wheat crop under variable N applications. Path analysis was computed to ascertain correlation (r) and regression coefficients (β) through multiple linear regression (MLR). The correlation (r) and regression (β) coefficients were further analyzed to interpret the contribution of grain yield components (GYC) and root traits (RT) towards the total yield in the form of direct effect (DE), indirect effect (IE) and total effect (TE). The PVC pipes trial was conducted during wheat cropping seasons i.e. November 2017 to May 2018 at Bio-field, Quaid-i-Azam University (QAU), Islamabad, Pakistan. A set of 100 historical cultivated wheat varieties of Pakistan were raised on sandy soil filled in 80cm tall PVC pipes of 10 cm radius mounted in open field. In this study grain yield components (GYC) and root traits (RT) were evaluated under high (HN; 120kg N/ha) and low (LN; 60 kg N/ha) nitrogen application. Present research outcome verified that wheat yield has been significantly affected by N fertilizer rate ($p < 0.001$). Reduction in N fertilizer application significantly decreased all quantity indices (GYC and RT) of yield. To improve the accuracy of selection for GY, a selection index involving the tiller number (β =0.28; r=.794** at HN and β =0.26 and r=.686** at LN) days to maturity (β=0.31; r=.792** at HN and β=0.16; r=.648** at LN), nitrogen use efficiency (β =0.27; r= .754**at HN and β =0.20 and r=.447** at LN) and root length (β=0.69; r=.709** at HN and β=0.70; r=.647** at LN) are recommended. These parameters showed high correlations and direct effects on GY under variable N application. Multiple linear regression (MLR) analysis by building path model is an effective way to predict improvement in grain yield as it showed the intensity of association between two or more yield related traits and indicated the relative importance of each trait.

3.2. Introduction

Application of nitrogen (N) fertilizer served as key contributor in increment of crop yield (Yadav et al., 2017). Though increased application of N fertilizer resulted in more yield but 60% of applied fertilizer is lost due to volatilization, leaching and runoff (Cameron et al., 2013). In wheat breeding program, improvement in grain yield is a major goal (Michel et al., 2019). Improvement in nitrogen response is one of the key strategies to increase crop yield in order to fulfill the ever increasing food demand of human populations around the globe (Ranjan et al., 2019). Basic genetic architecture of grain yield can be determined in a better way by studying grain yield components such as plant height, leaf area, spike length, thousand grain weight etc. This provides wheat breeders with an opportunity to produce high yielding cultivars with preferred combinations of yield components (Khan & Dar, 2010). Besides computation of correlation coefficient between grain yield and its component, path analysis can also be computed to predict and measure contribution of an independent variable (grain yield component) to dependent variable (grain yield). A regression coefficient (β) also known is as path coefficient basically measures the direct effect of one parameter (independent variable) upon another parameter (dependent variable) thus separates correlation coefficient into indirect and direct effects (Dewey & Lu, 1959; Ojha et al., 2018). MLR analysis of grain yield components and root traits, is an accurate tool to evaluate grain yield under variable N supply.

Nitrogen is acquired by most crops predominately in the form of nitrate (NO^{3-}), which is highly mobile anion due to its soluble nature (Cassman et al., 2002; van Grinsven et al., 2015). Nitrate capture is one of the most accessible breeding approaches which helps to increase NUE by improving N uptake. As roots serve as immediate contact points with soil solutions for plants, so different root traits are a primary focus of breeders to improve nitrate capture (Foulkes et al., 2009a; Palta et al., 2007). Water and nutrient uptake efficiency in different crops including rice, maize and wheat can be improved through selection of superior root architectures such as root length (RL), root biomass (RBM), lateral root dispersion (LRD), root surface area (RSA), root density (RD) and root mean number (RMN)(Paez-Garcia et al., 2015). Genetic progress to explore traits related to root architecture is limited due to difficulties in phenotyping of these traits at large scale. Advancement in root phenotypic screening techniques such as digital imaging, hydroponics, rhizotrons and pot screening have few limitations (Manschadi et al., 2006). PVC pipe screening provides a natural, uniform and an efficient medium for root growth. It facilitates wheat crop to maintain its intact root architecture compared to field conditions. Researchers can analyze root surface architecture more

efficiently through scanning or digital imaging from intact roots harvested from PVC pipes. Therefore the present experiment was conducted to study the effect of different N application rates on root and yield related traits of wheat varieties in a PVC pipe trial.

Few studies have reported the relationship between variable N application regimes and wheat grain yield through different scales at specific locations using several models (Dewey & Lu, 1959; Nazmi, 2013; Suleiman et al., 2014; Valkama et al., 2013). But no scientific study have reported the relationship between grain yield components and root traits with grain yield at different N-levels through path models using multiple linear regression including complete description of direct, indirect and total effects. The present study attempted to identify the major grain yield components and root traits and their level of contribution for yield maximization under variable N supplies through multiple linear regression and built their path model using LISREL software. It computes multiple linear regression (MLR) to show the interaction between independent (grain yield components and root traits) and dependent (grain yield) variables in the form of direct effect (DE), indirect effect (IE) and total effect (TE).

3.3. Materials and Methods

3.3.1. PVC pipe experiment, soil properties and weather data

The PVC pipes trial was conducted during wheat cropping seasons i.e. November 2017 to May 2018 at Bio-field, Quaid-i-Azam University (QAU), Islamabad, Pakistan. A set of 100 historical cultivated wheat varieties of Pakistan (detailed pedigree in Appendix 2.2) were raised on sandy soil filled in 80cm tall PVC pipes of 10 cm radius mounted in open field. Sandy loam soil (with 2:1 ratio of soil and sand respectively) used for the experimentation was first dried under the sun and then sieved properly. Random samples of soil were repeated taken in order to determine physico-chemical properties of the soil following Chen & Ma (2001). Soil EC, pH along with silt, clay and textural class were determined by making 10:1 w/v suspension of soil to d.H_2O through hydrometer method (Bouyoucos, 1936) as shown in Table 3.1. Available K, P and N were estimated through AB-DTPA method as shown in Table 3.1 (Soltanpour & Schwab, 1977). The sieved soil and sand mixture was then filled in PVC pipes at rate of 4kg per pipe.

Table 3.1. Physical and chemical properties of experimental soil.

Parameters	Unit	Mean±SD	Range
Soil texture	-	Loam	Loam
EC	dS/m	0.38±0.27	0.36-0.52
Soil pH	-	7.07±0.16	7.99-8.11
Clay	%	19.27±4.66	14.9-20.12
Silt	%	34.13±3.61	32.21-38.43
Sand	%	51.25±3.49	48.82-53.17
K	mg/kg	147.51±3.65	151-160
PO_4^{2-}-P	mg/kg	4.02±0.16	2.13-4.51
NO_3^{1-}-N	mg/kg	5.07±0.13	4.18-5.18

Temperature and humidity data of experimental site at different wheat growth stages was obtained from the "Pakistan Meteorological Department (PMD)", located in close vicinity (Figure 3.1).

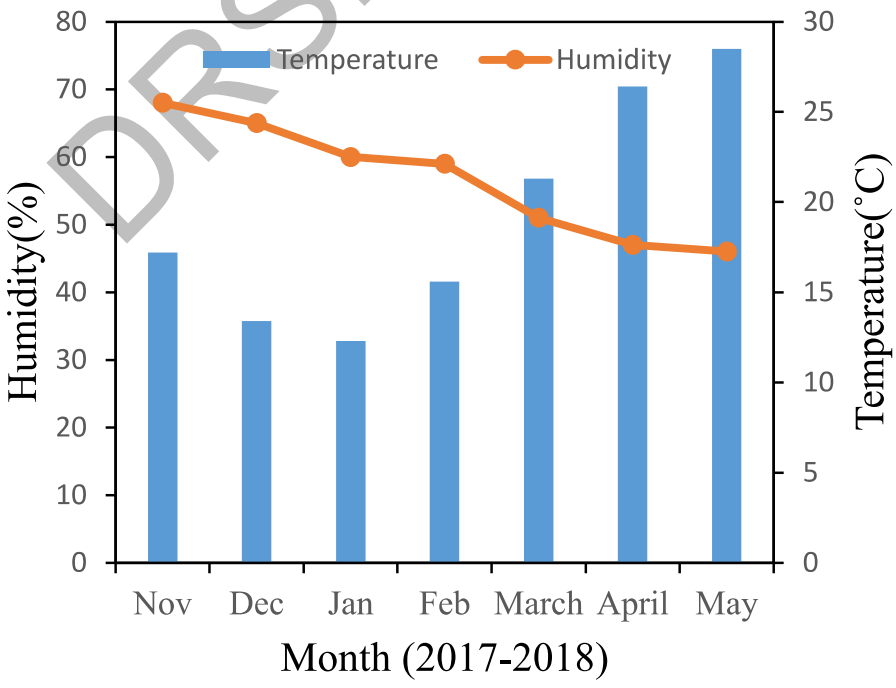

Figure 3.1. Weather data for the experimental period (November, 2017- May, 2018).

3.3.2. Experimental design and treatments

Three uniform seeds of each variety were surface sterilized and sown in individual PVC pipes. Detailed pedigree of these wheat varieties is given (Appendix 2.1) and plant material was obtained from Bioresources Conservation Institute (BCI), NARC, and Islamabad. For high and low nitrogen treatments i.e. HN and LN, nitrogen in the form of urea fertilizer was added in two split doses (half at time of sowing and remaining at tillering stage) at rates of 120 or 60 kg N/ha respectively. Potassium and phosphorous fertilizers in the form of potassium sulfate (K_2SO_4) and single super phosphate (Ca $(H_2PO_4)_2$) were added at the rate of 60 kg/ha to ensure good plant vigor. Triplicate PVC pipes for each treatment were arranged in a randomized complete block design (RCBD). Thinning of plant material from individual PVC pipe was done at three leaf stage leaving one plant per pipe. The plants were watered after an interval of two days throughout the experimental period to avoid effects of drought stress. The crop was harvested at physiological maturity on 13th May, 2018.

3.3.3. Parameter measurements

In the present study grain yield components and root traits were evaluated and measured. These includes chlorophyll content (CHL), plant height (PH), flag leaf area (FLA), days to maturity (DM), tillers number (TN), spike length (SL), spikelets per spike (SPS), thousand kernel weight (TKW), nitrogen use efficiency (NUE) grain yield (GY), root length (RL), root surface area (RSA) and root mean number (RMN). Chlorophyll content (CHL) was measured from flag leaf using a chlorophyll meter (Minolta SPAD-502: Minolta Camera Co., Tokyo, Japan) and an average reading was calculated from three biological replicates at anthesis stage. Flag leaf area (FLA) was calculated according to (Bavec et al., 2007);

$$\text{FLA } (cm^2) = \text{length of flag leaf(cm)} \times \text{width of flag leaf (cm)} \times 0.725 \ldots\ldots \text{ (Eq. 3.1)}$$

Nitrogen use efficiency (NUE) was calculated following Foulkes et al., (2009a); Moll et al., (1982);

$$\text{NUE } g/g = \text{Plant dry weight (g)} / \text{N supplied per plant (g)} \ldots\ldots \text{ (Eq. 3.2)}$$

After harvesting the crop at maturity, the roots of each cultivar were carefully removed from the PVC pipe soil to harvest roots in an intact form. We then transferred the roots to nylon bags (0.15 mm) and submerged in water for 30 minutes to remove the soil as previously described (Aziz et al., 2017; Palta et al., 2007). We measured the root length (RL), root surface area

(RSA) and root mean number (RMN) as root morphological descriptors using GIA Roots software (Alahmad et al., 2019; Galkovskyi et al., 2012).

3.3.4. Statistical analysis

To find out the individual and combined effects of nitrogen treatments and wheat varieties on different phenotypic traits under investigation, two-way analysis of variance (ANOVA) was performed using Statistica (Version 7.0; Stat Soft Inc., USA). To determine the intensity of linear relationship between the independents and dependent variables, Karl Pearson's coefficient of correlation was calculated through SPSS (Version 24.0) software.

3.3.5. Multiple linear regression

To test the potential interactions between the independents and dependent variables, the multiple linear regression (MLR) analysis was performed by the LISREL (Version 8.80) software (Bentler & Wu, 2002; Kline & Klammer, 2001; Nazmi, 2013). The observed phenotypic correlation between different variables can be decomposed into two parts through path models i.e. direct effect (DE); from independent variable (*x*) to dependent variable (*y*) and indirect effect (IE) from intermediate variables to dependent variables. One of the variables under study (GY in present case) was considered as dependent variable (effect) which is affected by many independent variables (causes). The total effect was calculated by the following equations which indicates the basic relationship between standardized regression coefficient or path coefficient (β) and correlation coefficients (r) as suggested previously (Dewey & Lu, 1959; Suleiman et al., 2014).

$$r(xy)=\beta(xy)+r(x1)\times\beta(1y)+r(x2)\times\beta(2y)+\ldots+rx(i-1)\times\beta(xy)\ldots\ldots \quad \text{(Eq. 3.3)}$$

Where i= 1, 2, 3, 4……n

Where, n is the number of independent variables (causes); r(*x*1) denotes correlation coefficients between causal factors 1 to *x*; β (1*y*) denotes the path coefficients between causal factor 1 and dependent variable (*y*) and so on. The indirect effect of i^{th} (independent) variable through j^{th} (intermediate) variable on y^{th} (dependent) variable was computed as β(i*y*) × r(*ji*).The sum of direct effect of x on y and products of all possible combinations of causal factors (*x*) with other remaining causal factors along with their regression/path coefficients (β) give total effect which is equal to correlation coefficient (r) between respective variable and dependent variable (Figure 3.2).

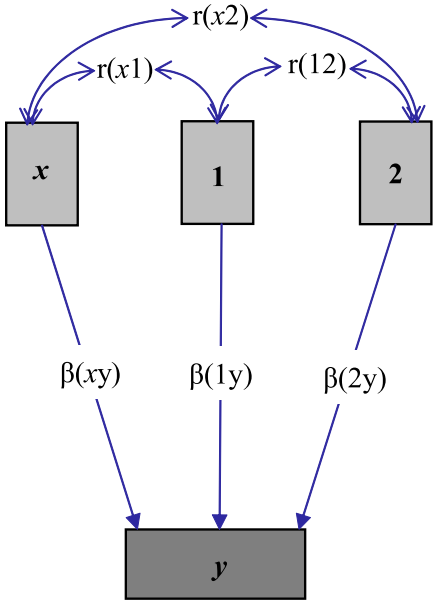

Figure 3.2. Path diagram illustrating the effect of independent variables on dependent variable. Bold arrow lines represent direct effect in form of regression/path coefficient (β) whereas curved arrows represent correlation (r) between independent variables. Note: Indirect effect is a combination of a direct effect and correlation coefficient between two independent variables.

3.4. Results

3.4.1. Evaluation of traits under HN and LN

We have evaluated different grain yield components (GYC) and root traits (RT) through different statistical tools to calculate range and mean performance of studied traits under high and low N supply (Table 3.2). All studied traits showed significant variation under both N fertilizer regimes. The mean for GY under HN was 10.383 g and under LN condition was 6.023 g. The GY range varied from 8.41 to 14.01g and from 4.33 to 11.04 g under HN and LN respectively. NUE ranges between 20.042 to 42.153 and 30.662 to 65.634 under HN and LN respectively. Overall mean value of NUE is less under HN compared to LN. While, CHL, PH, FLA, DM, TN, SL, SPS, TKW were higher under HN condition as compared to LN. Root traits including RL, RSA and RMN showed more variations under LN condition compared to HN. All traits except RMN were significant ($p > 0.01$) for the N levels and non-significant for varietal response except RL (Table 3.2).

Table 3.2 . Descriptive statistics and analysis of variance for agronomic and root traits of 100 wheat varieties under high (120 kg N/ha) and low (60 kg N/ha) nitrogen supply.

Traits	**Mean±SD** units?		**Range**		**Sources of Variation**		
	HN	**LN**	**HN**	**LN**	**N-levels**	**Varieties**	**Interaction**
GY	10.383±1.219	6.023±1.139	8.41-14.01	4.33-11.04	0.001***	0.528	0.535
CHL	46.711±3.494	42.174±4.33	40.334-54.734	33.084-51.8	0.002**	0.644	0.794
PH	72.725±5.268	66.93±6.098	63.5-87	54-80.88	0.007**	0.879	0.885
FLA	15.01±3.442	12.204±3.617	8.284-28.5	5.327-23.227	0.01**	0.723	0.835
DM	140.805±3.799	137.74±3.643	126-147	126.5-146	0.009**	0.752	0.835
TN	2.725±0.984	3.56±1.088	1-6	1-7	0.003**	0.383	0.466
SL	9.049±1.19	7.79±1.101	6.8-14.2	5-9.7	0.001***	0.444	0.526
SPS	17.72±2.284	15.16±2.558	11-23	8-21	0.001***	0.332	0.36
TKW	36.479±5.089	33.093±5.001	26.3-49.479	21.36-45.322	0.03*	0.832	0.966
NUE	28.846±4.823	48.027±7.535	20.042-42.153	30.662-65.634	0.001***	0.722	0.784
RL	47.099±8.191	54.524±9.767	27.21-71.051	34.745-87.247	0.001***	0.033*	0.497
RSA	5.519±0.535	5.539±0.539	3.354-6.906	4.354-7.906	0.003**	0.383	0.466
RMN	9.65±4.039	9.67±4.088	5-19	5-20	0.386	0.232	0.373

*GY=Grain yield, CHL=Chlorophyll, PH=Plant height, FLA=Flag leaf area, DM=Days to maturity, TN=Tiller number, SL=Spike length, SPS=Spikelets per spike, TKW=Thousand kernel weight, NUE=Nitrogen use efficiency, RL=Root length, RSA=Root surface area and RMN=Root mean number *Significant at $p \leq 0.05$, **Significant at $p \leq 0.01$, ***Significant at $p \leq 0.001$, without asterisk means non – significant.

3.4.2. Phenotypic correlation coefficients

3.4.2.1. Correlation between grain yield component (GYC) and grain yield (GY) under HN and LN

Phenotypic correlation coefficients between grain yield components (GYC) and grain yield (GY) were significant for the majority of traits (Table 3.3). The correlation coefficient between CHL and GY were at significance level of 0.01 under both N application rate with r value of .752** and .445** respectively. Plant height showed significant correlation with grain yield (r=.752** and .445*) under HN and LN, respectively. Correlation coefficients of FLA at high and low N treatment were .614** and .561** with GY respectively. Days to maturity showed significant correlation with GY at r=.792**at HN and .648** at LN. Tiller number per plant (TN) exhibited significant correlation (at 0.01 level of significance) in both N treatments which may be due to extended tillering period as a result of conducive field conditions (lower temperature) throughout the vegetative phase. While SL and SPS showed significant correlation at 0.01 level as r value equal to .721** and .754** under HN respectively. Both these traits also showed significant correlation with GY under LN. Thousand kernel weight (TKW) correlated non-significantly with GY at HN while significantly at LN. The correlation coefficient between TKW with GY were in opposite directions (r=.577** at LN) and (r=.179 at HN). NUE is significantly correlated with GY under both N treatment with r=.754** under HN and r=.477** under LN. Non-significant correlation coefficients were shown between PH, FLA, SPS with CHL and TKW with PH under LN treatment. Under high N supply, non-significant correlation were shown by TKW with PH, FLA, DM, TN, SL and NUE. While non-significant and negative correlation were shown by NUE with CHL and PH with r=-.001 and r=-.008 under low N supply respectively. Negative correlation coefficient was observed between TKW and SPS under high N supply (Table 3.3).

Table 3.3. Correlation coefficients for grain yield (GY) and yield components under different nitrogen supply.

	CHL	PH	FLA	DM	TN	SL	SPS	TKW	NUE	GY
CHL		.600**	.672**	.751**	.641**	.692**	.646**	.210*	.684**	.752**
PH	.121		.847**	.671**	.519**	.524**	.649**	.089	.830**	.562**
FLA	.171	.163		.660**	.539**	.594**	.594**	.117	.798**	.614**
DM	.497**	.252*	.385**		.698**	.670**	.645**	.116	.716**	.792**
TN	.545**	.319**	.310**	.604**		.753**	.683**	.038	.655**	.794**
SL	.509**	.339**	.320**	.619**	.550**		.610**	.119	.632**	.721**
SPS	.123	.564**	.406**	.475**	.498**	.479**		-.012	.800**	.745**
TKW	.467**	.118	.327**	.641**	.499**	.438**	.338**		.134	.179
NUE	-.001	-.008	.275**	.199*	.310**	.200*	.220*	.306**		.754**
GY	.445**	.277**	.561**	.648**	.686**	.550**	.557**	.577**	.447**	

Correlation coefficients for high (120 kg N/ha) and low (60 kg N/ha) are shown in upper and lower panels respectively. CHL=Chlorophyll content, PH=Plant height, FLA=Flag leaf area, DM=Days to maturity, TN=Tiller number, SL=Spike length, SPS=Spikelets per spike, TKW=Thousand kernel weight and NUE=Nitrogen use efficient** means Correlation is significant at the 0.01 level, * mean Correlation is significant at the 0.05 level, without asterisk means non-significant at 0.05 level.

3.4.2.2. Correlation between root traits (RT) and grain yield (GY) under HN and LN

A correlation coefficient between root parameters and grain yield is presented for LN and HN condition in Table 3.4. In the present investigation, under HN environment (above diagonal), RL has a positive and significant correlation with GY (r=.709**) and non-significant correlation with RSA (r=.024) and RMN (r=.24) respectively. RL showed negative and non-significant correlation with RSA and RMN under low N supply with r equal to -.120 and -.117 respectively. RSA showed significant correlation with GY under both HN and LN with same correlation coefficient value of .265**. Similarly, RMN showed same correlation coefficient value of .176 with GY under both N treatment but it is positive and non-significant. Correlation of RL with GY under low N application rate was significant (r=.647**). RMN and RSA are correlated with each other at significance level of 0.05 with r=.254* under both HN and LN (Table 3.4).

Table 3.4 . Correlation coefficients for root parameters under different nitrogen supply.

	RL	RSA	RMN	GY
RL		.074	.024	.709**
RSA	-.120		.254*	.265**
RMN	-.117	.254*		.176
GY	.647**	.265**	.176	

Correlation coefficients for high (120 kg N/ha) and low (60 kg N/ha) are shown in upper and lower panels respectively. RL=Root length, RSA=Root surface area and RMN=Root mean number** means Correlation is significant at the 0.01 level, * mean Correlation is significant at the 0.05 level, without asterisk means non-significant at 0.05 level.

3.4.3. Multiple linear regression (MLR)

When more traits are considered, the indirect association between these traits become less obvious, more complex and perplexing to some extent. To address these problems, path analysis could be used which can untangle the direct and indirect causes of association between the traits along with accurate measurement of relative importance of each causal factor/trait. Multiple linear regression (MLR) analysis was conducted to build the path model using LISREL (Version 8.80) software to investigate the relationships of GYC and RT with GY at HN and LN through LISREL software. The correlation coefficient (r) and regression or path coefficient (β) of these models were presented in (Figure 3.3 and 3.4 for GYC) and (Figure 3.5A and 3.5B for RT) at HN and LN respectively.

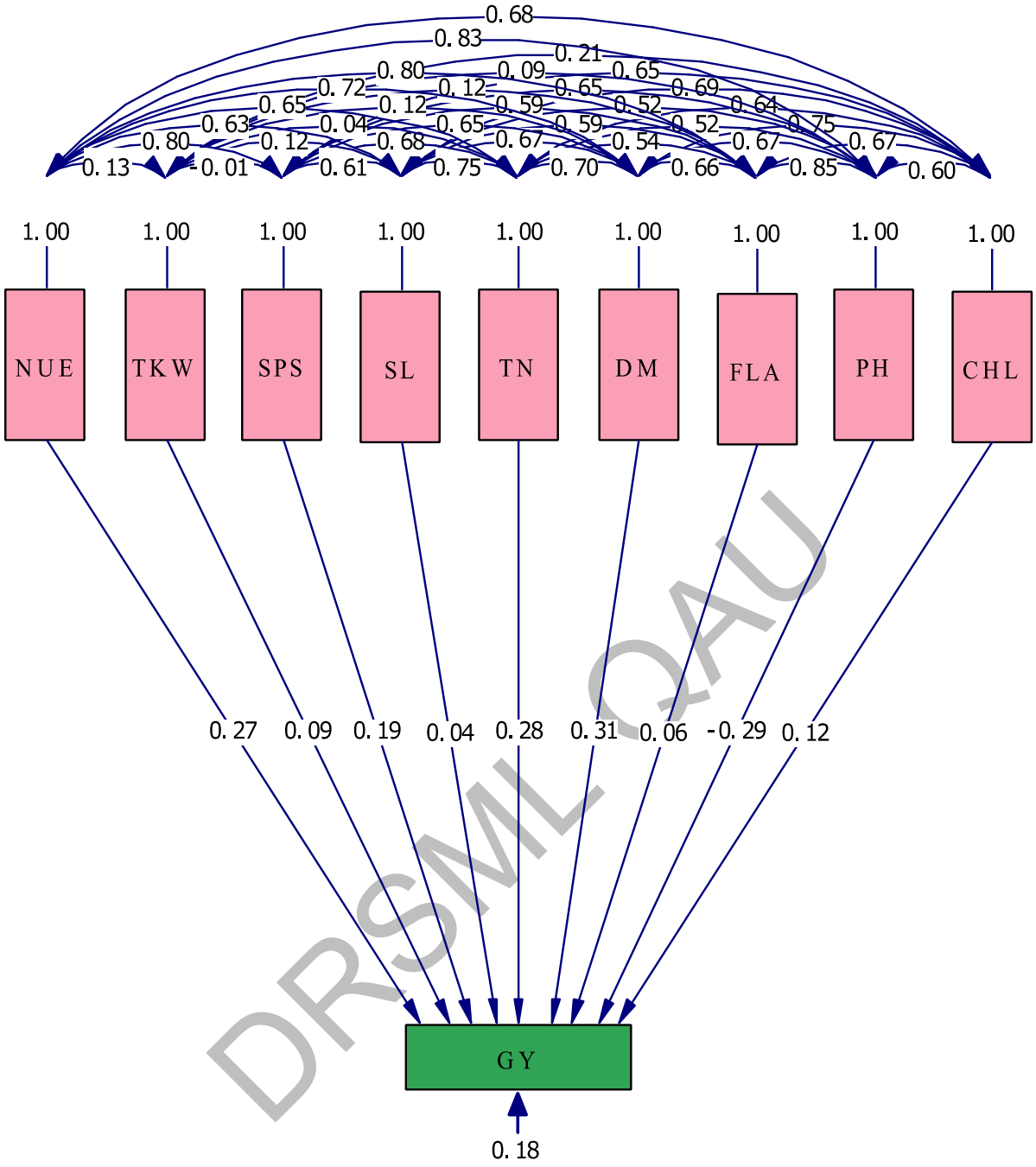

Figure 3.3. Path model illustrating interrelationships among the grain yield components (GYC) and grain yield (GY) under high (120 kg N/ha) nitrogen supply. The correlation coefficient (r) between GYC were present in upper part of the umbrella between curved arrows and regression or path coefficients (β) were present between the center of the bold arrows joining GYC with GY. Note: the nomenclature used in the figure is same as in Table 3.2.

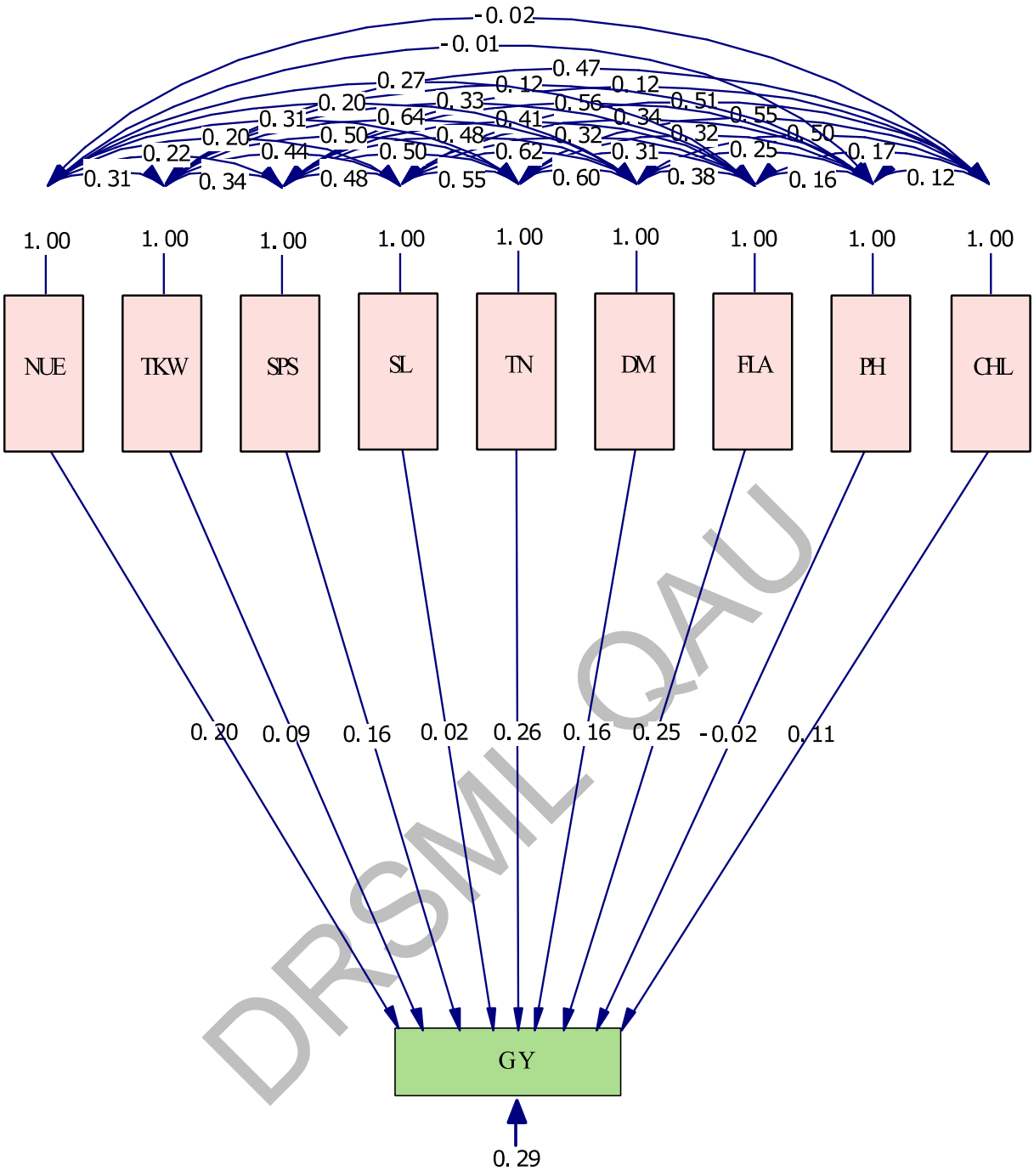

Figure 3.4. Path model illustrating interrelationships among the grain yield components (GYC) and grain yield (GY) under low (60 kg N/ha) nitrogen supply. The correlation coefficient (r) between GYC were present in upper part of the umbrella between curved arrows and regression or path coefficients (ß) were present between the center of the bold arrows joining GYC with GY. Note: the nomenclature used in the figure is same as in Table 3.2.

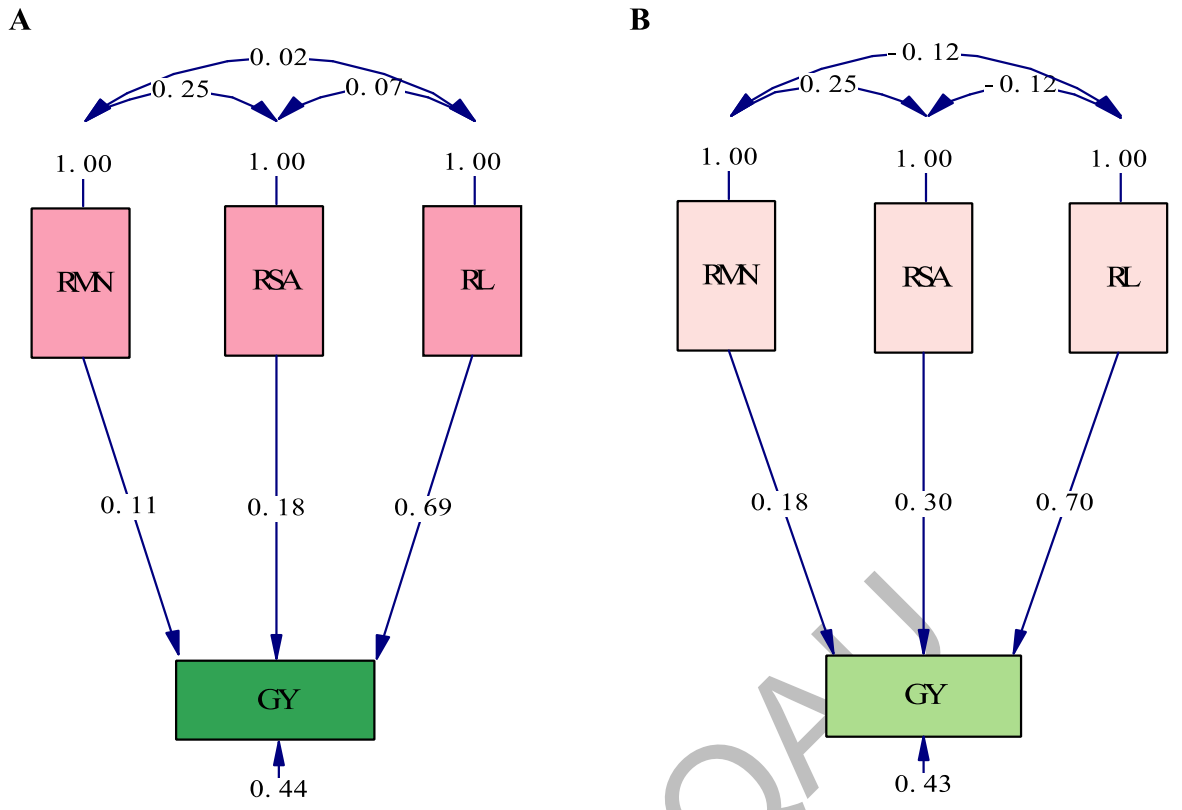

Figure 3.5. Path models illustrating interrelationships among the root traits (RT) and grain yield (GY). A: under high (120 kg N/ha), B: low (60 kg N/ha) nitrogen supply. The correlation coefficient (r) between RT were present in upper part of the umbrella between curved arrows and regression or path coefficients (β) were present between the center of the bold arrows joining GYC with GY. Note: the nomenclature used in the figures is same as in Table 3.2.

Through MLR analysis, it was observed that all GYC and RT showed statistically significant variations at both HN and LN except TKW. According to R square statistic, GYC showed 18 % variance at HN and 29% variance at LN for the estimation of GY (Figure 3.3 and 3.4). Root traits (RT) showed 44% variance at HN and 43% at LN to GY (Figure 3.5A and 3.5B). In terms of the relative contribution of the independent variables to dependent variable, it was observed that in the case of GYC, the DM, NUE and TN showed highest contribution across the model at HN whereas FLA, NUE and TN at LN (Figure 3.3 and 3.4) respectively. While in case of root traits, RL showed highest contribution to the GY at both HN and LN (Figure 3.5A and 3.5B).

The positive sign of the regression or path coefficients (β) pertaining to these variables indicates that there was a positive direct relationship between GY and all GYC except PH, which was negatively correlated to GY. If CHL, PH, FLA, DM, TN, SL, SPS and NUE increased and PH decreased, the GY will increase at both N treatment. Whereas no significant

variation was observed for TKW at both N-levels. While in the case of RT, all independent variables (RL, RSA and RMN) were positively correlated with dependent variable (GY) at both HN and LN. All traits including RL, RSA and RMN contributed less to GY at HN compared to LN as root architecture modified at LN (60kg N/ha) to better cope with N stress conditions.

3.4.4. Direct, Indirect and Total Effects of GYC and RT on GY

In order to precisely understand the relative contribution of each GYC and RT on the GY presented in the path models, we analyzed their association in the form of direct, indirect and total effects in Table 3.5 and 3.6 for GYC and RT respectively. It is an effective method to identify the mutual association between traits and their relative contribution to the grain yield under high and low nitrogen conditions. It indicated the relative importance of each trait that could allow wheat breeders to gain insight on grain yield potential. It was observed that total effect (TE) with sign and magnitude was similar to the correlation coefficient (r) between respective parameter and GY. The TE of all grain yield components were higher under HN as compared to LN with the exception of FLA and TKW. TKW showed a non-significant correlation with GY (0.179) and similarly low direct effect (0.087) at HN.

Table 3.5. Direct, indirect and total effects of grain yield components (GYC) on grain yield (GY) under high (120 kg N/ha) and low (60 kg N/ha) nitrogen supply.

Effects	Indirect via CHL	Indirect via PH	Indirect via FLA	Indirect via DM	Indirect via TN	Indirect via SL	Indirect via SPS	Indirect via TKW	Indirect via NUE	Total indirect effect (IE)	Direct effect (DE)	Total Effect (TE)
High Nitrogen supply (HN = 120 kg N/ha)												
CHL	_	-0.175	0.043	0.230	0.178	0.028	0.123	0.018	0.187	0.632	0.120	0.752
PH	0.072	_	0.054	0.206	0.144	0.021	0.123	0.008	0.226	0.855	-0.292	0.563
FLA	0.081	-0.247	_	0.202	0.150	0.024	0.113	0.010	0.218	0.550	0.064	0.614
DM	0.090	-0.196	0.042	_	0.194	0.027	0.123	0.010	0.195	0.485	0.306	0.792
TN	0.077	-0.152	0.035	0.214	_	0.030	0.130	0.003	0.179	0.516	0.278	0.794
SL	0.083	-0.153	0.038	0.205	0.209	_	0.116	0.010	0.172	0.681	0.040	0.722
SPS	0.077	-0.190	0.038	0.198	0.190	0.025	_	-0.001	0.218	0.555	0.190	0.746
TKW	0.025	-0.026	0.007	0.035	0.010	0.005	-0.002	_	0.037	0.092	0.087	0.179
NUE	0.082	-0.242	0.051	0.219	0.182	0.026	0.152	0.012	_	0.481	0.273	0.755
Low Nitrogen supply (LN = 60 kg N/ha)												
CHL	_	0.000	0.042	0.078	0.140	0.011	0.019	0.042	0.000	0.332	0.112	0.445
PH	0.014	_	0.040	0.040	0.082	0.007	0.089	0.011	-0.002	0.280	-.004	0.277
FLA	0.019	-0.001	_	0.061	0.080	0.007	0.064	0.029	0.056	0.315	0.246	0.561
DM	0.056	-0.001	0.095	_	0.155	0.013	0.075	0.058	0.040	0.491	0.157	0.649
TN	0.061	-0.001	0.076	0.095	_	0.012	0.078	0.045	0.063	0.429	0.257	0.687
SL	0.057	-0.001	0.079	0.097	0.142	_	0.075	0.039	0.040	0.529	0.022	0.551
SPS	0.014	-0.002	0.100	0.075	0.128	0.010	_	0.030	0.044	0.400	0.157	0.557
TKW	0.053	0.000	0.080	0.101	0.128	0.010	0.053	_	0.062	0.486	0.090	0.577
NUE	0.000	0.000	0.068	0.031	0.080	0.004	0.035	0.028	_	0.245	0.202	0.448

*CHL=Chlorophyll content, PH=Plant height, FLA=Flag leaf area, DM=Days to maturity, TN=Tiller number, SL=Spike length, SPS=Spikelets per spike, TKW=Thousand kernel weight and NUE=Nitrogen use efficient

The total effect of all root traits on GY is lower at HN compared to LN (Table 3.6). The reason behind this variation is that all traits related to root architecture are polygenic in nature and vastly impacted by environments thus have tough and stringent selection efficiencies.

Table 3.6 . Direct, indirect and total effects of root traits (RT) on GY under high (120 kg N/ha) and low (60 kg N/ha) nitrogen supply.

Effects	Indirect via RL	Indirect via RSA	Indirect via RMN	Total Indirect effect (IE)	Direct effect (DE)	Total effect (TE)
High Nitrogen supply (HN = 120 kg N/ha)						
RL		0.014	0.003	0.016	0.692	0.709
RSA	0.051		0.028	0.080	0.185	0.265
RMN	0.017	0.047		0.064	0.112	0.176
Low Nitrogen supply (LN = 60 kg N/ha)						
RL		-0.036	-0.021	-0.057	0.704	0.647
RSA	-0.084		0.046	-0.038	0.303	0.265
RMN	-0.082	0.077		-0.005	0.181	0.176

*RL=Root length, RSA=Root surface area and RMN=Root mean number

3.5. Discussion

Current research work computes multiple linear regression (MLR) to show the interaction between independent (grain yield components and root traits) and dependent (grain yield) variables in the form of direct effect (DE), indirect effect (IE) and total effect (TE). This approach provides wheat breeders with an opportunity to produce high yielding cultivars with preferred combinations of yield components. In the past, some studies also reported path models to predict relationship between agronomic traits in wheat (Dewey & Lu, 1959; Ojha et al., 2018; Suleiman et al., 2014).

Most of the agronomic traits including grain yield are quantitative in nature that is controlled by action and interaction of several component parameters. The GY, SL, SPS, NUE and RL were highly affected by the N levels with P≥0.001, indicating significant differences in the responses of the varieties to the different N levels for these parameters (Table 3.2). Such a strong interaction between varieties and N environment was already reported and suggests a separate breeding program for selection and improvement of varieties in term of N response

(Ranjan 2018). The large variations in all grain yield components and root parameters under variable N supply indicated the absence of direct selection on the basis of one or two traits and strongly recommends wheat breeders for further yield consolidation through exploitation of these variations without any harmful footprints on the surrounding environment.

Chlorophyll content showed a significant variation in the current study (Table 3.2). Nitrogen is the main constituent of chlorophyll pigment and proteins (Adhikari et al., 1999). Therefore, chlorophyll content measured from the flag leaf at anthesis stage was significantly affected by different N-levels (Islam et al., 2014; Ranjan et al., 2019). Plant height has a significant correlation with all other yield related traits at both N levels except TKW under both HN and LN and FLA under LN. Differences in genetic makeup of different varieties is one of main attributes responsible for variation in PH. Results of present study were in line with reported data that higher levels of N significantly improved the plant height as more available nitrogen is responsible for this increment (Mattas et al., 2011; Sultana et al., 2013). Days to maturity showed a significant correlation with GY at r=.792**at HN and .648** at LN. A similar finding has been reported in the past by Suleiman et al., (2014). In our work, tiller number showed a maximum direct effect on grain yield under both N levels. This inference was supported by past studies; stated that extended tillering period resulted in more grain yield (Xie et al., 2016). It was evident that the proportional and accurate N application rate increases grain yield of wheat crop through increment in SL and SPS. It was previously reported that nitrogen fertilizer increased the SL during the two years of the field experiment (Fischer, 1985; Mosanaei et al., 2017).

The correlation coefficients of TKW with GY were in opposite directions (r=.577** at LN) and (r=.179 at HN). These results were not in agreement with previous findings on relationship between 1000-grain weight with GY at different N-level (Linina & Ruza, 2018). NUE is significantly correlated with GY under both N treatment with r=.754** under HN and r=.477** under LN (Table 3.3). A reduction in artificial N fertilizer rate greatly suppress the wheat NUE. In previous studies, it has been reported that NO_3-N levels of soil were directly correlated with crop yield (Miao et al., 2015). These results of present studies verified the work of other researchers that plant N concentration increased with cumulative trend of N fertilizer application (Garrido-Lestache et al., 2005). The non-significant relationship between PH, FLA, SPS with CHL and TKW with PH under LN, may be due to competitive reasons among them as the biological yield is generally determined by leaf area, storage capacity of kernels and stem carbohydrates (Ju et al., 2009).

The current study also validated the correlation of root parameters with grain yield under varying N-levels and concluded that the root length showed significant and maximum correlation with GY compared to other root traits. Significant variations among root parameters were reported in past (Petrarulo et al., 2015). Present study reported that root surface area (RSA) and root mean number (RMN) have less significant direct impact on wheat yield under both N-levels (Table 3.4). While previous reports stated that most crop varieties responded to optimum nutrient level by producing shallow but dense roots in order to absorb a greater fraction of the available nutrients thus resulting in healthy plants with more biomass (Ehdaie et al., 2010). Another study by Foulkes et al., (2009a), reported that in wheat under limited N supply, roots responded to applied nitrogen by increment in number of root axis, depth and density of roots along with root longevity at post-anthesis stage.

All agronomic traits are positively or negatively correlated with each other. The path diagrams showed, in essence, that GY is the result of grain yield components (CHL, PH, FLA, DM, TN, SL, SPS, TKW and NUE) and root traits (RL, RSA, RMN) in this study (figure 3.3, 3.4, 3.5A and 3.5B). All variables are themselves interrelated; consequently, each parameter influences GY through direct contribution and indirectly in combination with the other parameters with which it has a correlation. The key advantage of a path analysis is that it deconstructs the phenotypic correlation coefficient and presents it in the form of direct and indirect effects, predicting the cause and effect relationship between the studied traits. Nitrogen fertilizer have regulated different growth indices i.e. GYC and RT to ensure better yield through direct and indirect contribution of these aforementioned traits.

3.6. Conclusion

Grain yield is a complex trait which is influenced by different environmental and genetic factors. Thus in a wheat breeding program, direct selection based on association of different agro-physiological traits with grain yield could be misleading. Correlation analysis basically measures the intensity of association between two or more traits but it does not indicate relative importance of each trait that could allow wheat breeders to gain insight on grain yield potential. Thus, further validation through path coefficient analysis which basically breaks down the correlation coefficient (r) into direct and indirect effects ultimately indicating the relative importance of each trait as independent cause on grain yield. Therefore, in the present study, correlation (r) and path (β) coefficients among grain yield components and root traits with grain yield were computed to use them as selection criteria for grain yield. However, based on the

results of path-coefficient analysis, it could be concluded that tiller per plant (TpP), days to maturity (DM), nitrogen use efficiency (NUE) and root length (RL) were the most important traits. Hence, these traits could be use as indirect selection criteria to improve grain yield under varying N-levels. This approach provides wheat breeders with an opportunity to produce high yielding cultivars with preferred combinations of yield components.

Chapter#4

Wheat Varietal Response to Relative SPAD Index (RSI) and Relative Normalized Difference Vegetation Index (RNDVI) under Variable Nitrogen Application and Terminal Heat Stress along with Yield Repercussion

Chapter # 4

Wheat Varietal Response to Relative SPAD Index (RSI) and Relative Normalized Difference Vegetation Index (RNDVI) under Variable Nitrogen Application and Terminal Heat Stress along with Yield Repercussion

4.1. Abstract

Nitrogen (N) deficiency and heat stress (HS) are major abiotic stresses that affect the quantity and quality of wheat grains. This study was conducted to examine wheat varietal response to RSI and RNDVI at the anthesis stage and their relationship to yield and yield-related traits under variable N supply and terminal heat stress. Twelve wheat varieties were evaluated in 2016–2017 and 2017–2018 at the National Agricultural Research Centre (NARC), Islamabad, Pakistan. The experiment was divided into three sets, i.e., N120 (120 kg N/ha), N60 (60 kg N/ha) and N0 (0 kg N/ha), based on the nitrogen fertilizer application. The physiological and yield-related parameters were recorded. Mean grain yield for all twelve varieties, averaged from two years data, ranged between 1655.0 and 3890.1 kg/ha. Maximum RSI (0.99), RNDVI (1.03) and GY (3890.9 kg/ha) were recorded for FSD-08, while AARI-11 showed minimum RSI (0.50), RNDVI (0.56) and GY (1396.40 kg/ha). In the present study, mean CTD was lower, at N0 (3.57 ˚C), followed by N60 (5.07 ˚C) and N120 (5.47 ˚C) on average for the two years of data. The strong positive correlation of RSI and RNDVI with grain yield at $R^2 = 0.73$ and $R^2 = 0.49$ suggest that these parameters can be used as efficient and precise selection criteria for identifying nitrogen-use-efficient wheat varieties under terminal heat-stress conditions. This work will help researchers to identify and develop nitrogen-use efficient and thermos-tolerant wheat cultivars by minimizing the negative impacts of heat stress at the anthesis stage.

4.2. Introduction

Wheat crop covers 17% of the world crop cultivated area and contributes to approximately 20% of the total calories in the human diet (Shiferaw et al., 2013a). It is a staple cereal crop for 40% of the world population (Shewry & Hey, 2015). Major constraints for wheat production are abiotic stresses, including low soil fertility, nutrient deficiency, heavy metal stress, moisture deficit, salinity stress, drought stress and heat stress (Mantri et al., 2012). Heat stress is one of the major challenges that significantly impacts wheat yield, and it occurs repeatedly during the cropping season (Ni et al., 2018). In the current climatic conditions, rising temperatures are a serious threat that can cause tremendous decreases in wheat production (Yadav et al., 2022). It

reduces crop yield through alterations in physiological processes, such as photosynthesis, protein denaturation, fatty acids accumulation, membrane thermostability, and starch synthesis. It also accelerates vegetative growth, ultimately leading to decreased grain filling duration (Tahir & Nakata, 2005; Zahedi & Jenner, 2003). One important strategy to overcome losses due to heat stress is the selection of heat-tolerant genotypes that could be better adapted to high temperature, thus maintaining the desired yield (Yang et al., 2002). Besides this breeding approach, wheat yield under heat stress could be maintained and improved through modified crop micro-climatic conditions, such as frequent irrigation, mulching and optimized nitrogen fertilization application (Kingra & Kaur, 2017).

The application of nitrogen fertilizer usually results in more above-ground biomass, seed production, flag leaf area and grain protein (Adnan et al., 2016). It is used for the synthesis of amino acids, signalling molecules, and storage molecules. It is also utilized in a number of metabolic processes (Sun et al., 2014). Thus, the use of nitrogen fertilizer significantly improves crop performance and yield-related traits under normal climatic conditions as well as results in higher canopy temperature depression (CTD) values under heat stress conditions (Ali, 2000; Elfadil et al., 2012; Modhej et al., 2012). Canopy temperature depression (CTD) is defined as the difference between crop canopy temperatures from the ambient temperature (Rosyara et al., 2008). It has a direct correlation with grain yield and other related traits, including NDVI, SPAD (special product analysis division) value, nitrogen-use efficiency (NUE) and biomass under a hot environment, including both rain-fed and irrigated crop cultivation areas (Elfadil et al., 2012). Under a climate change scenario, SPAD and NDVI demonstrated a highly significant relationship with grain and yield-related traits, proving their reliability as indicators of nitrogen deficiency and selection of superior wheat varieties to ensure food security (Kizilgeci et al., 2021).

Varietal response for nitrogen-use efficiency and canopy temperature depression has already been reported and verified. However, currently, little information is known about varietal response to different N application rates under terminal heat stress and maintaining crop yield by lowering canopy temperature along with improvements in related agronomic and physiological traits. Therefore, this study aimed to investigate the varietal response for available nitrogen, categorizing wheat varieties as N-use efficient, moderately N-use efficient, moderately N-use inefficient and N-use inefficient, on the basis of the relative SPAD index (RSI), relative normalized difference vegetation index (RNDVI) and nitrogen agronomic

efficiency (NAE). Additionally, the present research work reported varietal differences in utilizing available N under dry and hot rain-fed environmental conditions of Pakistan.

4.3. Materials and methods

4.3.1. Experimental site, soil properties, weather data and plant material

The field experiments were conducted during two consecutive wheat cropping seasons, i.e., from November 2016 to May 2017 and from November 2017 to May 2018 at the National Agricultural Research Centre (NARC), Islamabad, Pakistan. At different growth stages of wheat, minimum, maximum and mean temperatures were obtained from the Pakistan Meteorological Department (PMD) which was located in close proximity to the experimental sites during both cropping seasons (Table 4.1).

Table 4.1 . Minimum, maximum and mean temperatures (◦C) for 2016–2017 and 2017–2018 at the National Agricultural Research Centre, Islamabad.

Period	First year (2016-17)			Second year (2017-2018)			Growth stage
	Min	Max	Mean	Min	Max	Mean	
November	7	21	14	3	20	11.5	Sowing/germination
December	4	22	13	-2	18	8	Vegetative
January	-5	12	3.5	0	17	8.5	Tillering
February	0	16	8	-2	16	7	Tillering/booting
March	-2	23	10.5	2	24	13	Heading/anthesis
April	4	28	16	4	26	15	Grain filling
May	10	28	19	9	28	18.5	Maturity

Table Information Sources: "Pakistan Meteorological Department (PMD)"

For soil analysis, samples from ten different sites of the field (n = 10) were collected and analysed to record soil parameters following Chen & Ma (2001). Available N, available K, and available P were estimated using the AB-DTPA method (Soltanpour & Schwab, 1977). EC, pH, clay percentage, silt percentage and textural class were recorded by making a 10:1 w/v suspension of soil to d.H2O using the hydrometer method as shown in Table 4.2 (Bouyoucos, 1936).

Table 4.2 . Physico-chemical properties of soil at the experimental site (n = 10).

Parameters	Unit	Mean±SD	Range
NO_3^{1-}-N	mg/kg	5.88±0.14	5.18-5.98
K	mg/kg	154.51±4.94	151-160
PO_4^{2-}-P	mg/kg	3.08±0.18	2.91-3.21
pH	-	8.07±0.12	7.99-8.11
EC	dS/m	0.48±0.07	0.39-0.54
Clay	%	17.51±3.25	14.9-19.92
Silt	%	37.05±3.46	34.21-39.52
Sand	%	49.25±2.89	46.82-51.36
Textural class	-	Loam	Loam

Twelve wheat varieties commonly cultivated due to their commercial significance in different provinces of Pakistan, i.e., Punjab, Khyber Pakhtunkhwa and Sindh, were selected. These varieties include FSD-08, NARC-09, PIRSBK-08, T-8, TD-1, PAKISTAN-13, AAS-11, CHAKWAL-50, GA-2002, INQILAB-91, SH-2002 and AARI-11. A detailed pedigree of these wheat varieties is given in Table 4.3, and the plant material was obtained from the Bioresources Conservation Institute (BCI), NARC, Islamabad.

Table 4.3. Detailed description of studied plant material.

S. No.	Variety name	Pedigree
1	FSD-08	PBW65/2*Pastor
2	NARC-09	INQALAB 91*2/TUKURU
3	PIRSBK-08	JUP/ALD'S'//KLT'S'
4	T-8	land races
5	TD-1	PITIC-62/FROND//MEXIPAK/3/PITIC-62/MAZOE-79-75-76
6	PAKISTAN-13	CMH84.3379/CMH78.578//MILAN
7	AAS-11	LU26/HD 2179
8	CHAKWAL-50	F6.74/BUN//SIS/3/VEE#7 or F6-74/BUN//SIS/3/VEE#7
9	GA-2002	NAI60/CB151//S949/3/MEXIPAK
10	INQILAB-91	V-1562//CHRC'S'/HORK/3/KUFRA-I/4/CARP'S'/BJY'S'
11	SH-2002	INQALAB-91/FINK'S'
12	AARI-11	OPATA/RAYON//KAUZ

Table information sources: "wheatpedigree.net" and Country-wide specific "Wheat Breeding Programs.

4.3.2. Experimental layout and treatments

Selected wheat varieties were planted in a randomized complete block design (RCBD) with split plot arrangement having three replications, while the net plot size was 8 × 2 m^2. The varieties grown in sub-plots were replicated in the field trials at different rates of N (urea) application from the main plots (no fertilization, optimum fertilization and full recommended fertilization at the sowing site). The experiment was divided into three sets, i.e., N120 (120 kg N/ha), N60 (60 kg N/ha) and N0 (0 kg N/ha), based on the application of N fertilizer. The urea fertilizers were applied as the source of nitrogen in three equal splits, i.e., before sowing, at the tillering stage and at the booting stage. Potassium (potassium sulphate) and phosphorous (single super phosphate) fertilizers were added at a rate of 60 kg/ha to ensure good plant vigour (Pask et al., 2012). The crop was harvested on 12 May 2017 in the first year and on 16 May 2018 in the second cropping year, at physiological maturity. All other agronomic practices such as weeding, irrigation, etc., were kept standard except for the application rate of the nitrogen fertilizer.

4.3.3. Phenotypic analysis

Phenotypic traits considered and evaluated in this study were: plant height (PH), tillers per plant (TpP), nitrogen agronomic efficiency (NAE), chlorophyll content in the form of relative SPAD index (RSI), canopy temperature as canopy temperature depression (CTD), normalized difference vegetative index (NDVI) as RNDVI, grains per spike (GpS), spike length (SL), thousand kernel weight (TKW), biological yield (BY), grain yield (GY) and harvest index (HI). Nitrogen-use efficiency (NUE) is calculated in terms of agronomic efficiency (kg/kg), which is GY per unit of nitrogen supply by following (Fageria & Baligar, 2005), and it was calculated as:

$$\text{NAE (kg/kg)} = \text{Gf (kg)} - \text{Gu (kg)}/\text{Na (kg)}/\text{Na applied} \quad \text{(Eq. 4.1)}$$

Where NAE is nitrogen agronomic efficiency, Gf is grain yield (GY) in fertilized plots, Gu is unfertilized plots, and Na is the amount of applied N fertilizer. The harvest index was calculated as the ratio of grain yield to biological yield, i.e.

$$\text{HI}=\frac{\text{GY}}{\text{BY}}\times 100 \quad \text{(Eq. 4.2)}$$

Where HI is harvest index, GY is grain yield, and BY is biological yield. Chlorophyll content (CC) was measured by using chlorophyll meter (Minolta SPAD-502: Minolta Camera

Co., Tokyo, Japan), and averages were reported in triplicate from flag leaf at the anthesis stage. The relative SPAD index was calculated as the ratio of the SPAD value on one treatment to that of the heavily fertilized treatment of the same variety in the same trial, i.e., treatment by following (Prost & Jeuffroy, 2007):

$$\text{SPAD index } (i, j) = \text{SPAD } (i, j) / \text{SPAD ref } (i) \quad \text{(Eq. 4.3)}$$

Where i is the variety and j is the nitrogen treatment. Crop vegetation index was assessed using the handheld Green Seeker (crop sensor) to take a reading of crop vigour (Gamon et al., 1995; Raun et al., 2002). The sensor emits transitory bursts of red (visible spectrum) and near infrared (NIR spectrum) light and records their reflected intensity from the plant. The Green Seeker displays the measured value as an NDVI reading, i.e., from 0.00 to 0.99, and the detected light strength is a direct indication of the nitrogen amount in the crop. The NDVI readings were taken from canopies of leaves at the anthesis stage. The NDVI was calculated by using the equation (Cao et al., 2012):

$$\text{NDVI} = (\text{NIR}_{\text{reflected}} - \text{Red}_{\text{reflected}}) / (\text{NIR}_{\text{reflected}} + \text{Red}_{\text{reflected}}) \quad \text{(Eq. 4.4)}$$

Where RNDVI of each variety was calculated as a ratio of NDVI at treatment to that of the heavily fertilized treatment of the same variety in the same experimental trial by following (Cao et al., 2012):

$$\text{RNDVI}(i, j) = \text{NDVI}(i, j) / \text{NDVI ref}(i) \quad \text{(Eq. 4.5)}$$

Where i is the variety, and j is the nitrogen treatment. Canopy temperature was measured at noon (13:00 to 14:00) in full sunshine with a handheld infrared thermometer (IRT; Everest Inter Science, INC, Tucson, AZ, USA) with 45◦ viewing angle at a horizontal line above the crop canopy to circumvent the perplexing effect of soil temperature (Elfadil et al., 2012). The IRT (infrared thermometer) senses radiation emitted from crop canopies. Readings were taken at the anthesis stage to measure terminal heat stress, while CTD was calculated by the following expression (Rosyara et al., 2008):

$$\text{CTD} = \text{Ambient Temperature (AT)} - \text{Canopy temperature (CT)} \quad \text{(Eq. 4.6)}$$

Readings of RSI, RNDVI and CTD were taken on the 5th day after the anthesis stage and during the grain-filling period, at the same time-point.

4.3.4. Statistical analysis

Two-way analysis of variance (ANOVA) was performed using Statistica Ver.7.0 (Stat Soft Inc., Tulsa, OK, USA) to find out the individual and combined effects of nitrogen treatments and wheat varieties on different phenotypic traits under investigation. Thus, based on the mentioned criteria, wheat varieties were classified as nitrogen-use efficient, moderately nitrogen-use efficient, moderately nitrogen-use inefficient, and nitrogen-use inefficient at an optimum N application rate (60 kg N/ha) by principal component analysis (PCA) using XLSTAT Version 2018 (Addinsoft). Further validation of PCA results was performed through the HACA (Hierarchical agglomerative cluster analysis) using Ward's linkage technique and Euclidean distance measure.

4.4. Results

4.4.1. Biplot analysis validates contrasting varieties for N response

In order to statistically validate the response of twelve wheat varieties under varied N application rates, biplot analysis was carried out on RSI, RNDVI and NAE values at an optimum N application rate, i.e., N60 averaged from two years of data by the PCA method using XLSTAT software. The biplot in Figure 4.1 shows the most varied wheat varieties, which account for the phenotypic variation in N response. In the PCA plot, the vectors represent agro-physiological traits, e.g., RSI, RNDVI and NAE, while their length indicated the variations of traits under consideration. The variation shown by two principal components was 78.69% (PC1) and 14.34% (PC2). From the PCA plot, it was inferred that these twelve varieties fell into four clusters and were categorized as N-use efficient, moderately N-use efficient, moderately N use-inefficient and N-use inefficient. FSD-08, PIRSBK-08, NARC-09 and T-8 are in one cluster and were positioned toward the RNDVI and RSI vectors, thus indicating impact of these traits on these four wheat varieties hence termed as nitrogen-use efficient varieties, since these parameters were good indicators of the contrasting responses of wheat varieties to N fertilizer application. These four varieties showed the highest mean RSI (0.99, 0.97, 0.94 and 0.93) and RNDVI (1.03, 1.00, 0.98 and 0.97) (Appendix 4.1). TD-1, AAS-11, PAKISTAN-13 and CHAKWAL-50 were grouped in the second cluster and were termed as moderately N-use efficient, as these were positioned in close proximity to the main axis, and these three agro-physiological vectors had moderate impact on all four varieties, with mid-ranged RSI (0.92, 0.85, 0.89 and 0.81) and RNDVI (0.95, 0.90, 0.92 and 0.85) values, as shown

in Appendix 4.1. Conversely, there were two wheat varieties, i.e., GA-2002 and INQILAB-91, in the third cluster, termed as moderately N-use inefficient, as these were positioned in the opposite direction to the RSI and RNDVI vectors but are in close proximity of the NAE vector, with high mean NAE values (4.69 and 8.36 kg/kg), as shown in Appendix 4.1. The fourth cluster in the PCA plot represented N-use inefficient wheat varieties, including SH-2002 and AARI-1, and these were positioned toward the NAE vector, indicating that these varieties exhibited higher NAE values. It can also be observed from Appendix 4.1 that these N-use inefficient varieties exhibited the lowest mean values of RNDVI (SH-2002; 0.65 and AARI-11; 0.56) and RSI (SH-2002; 0.56 and AARI-11; 0.50) but revealed the highest mean values for NAE (SH-2002; 7.95 kg/kg and AARI-11; 5.66 kg/kg).

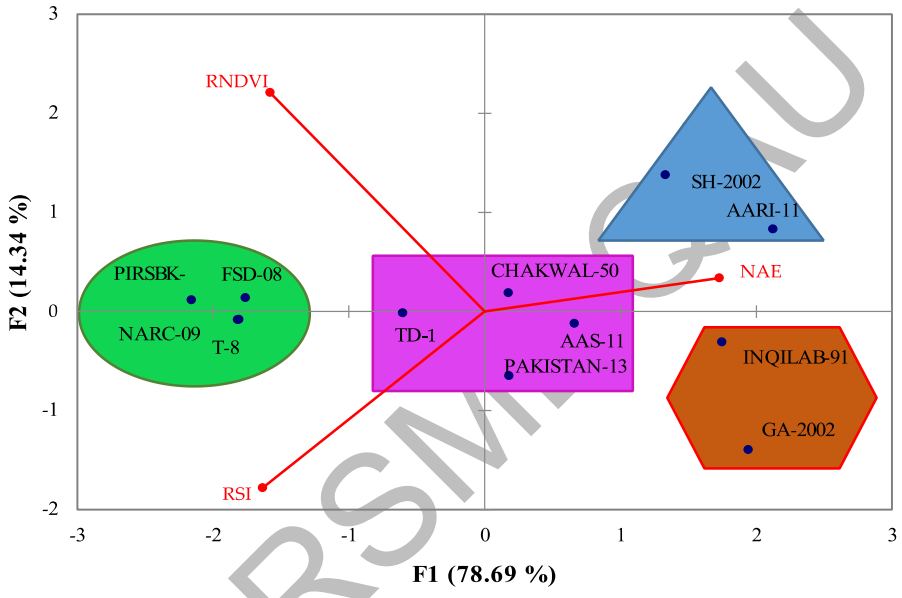

Figure 4.1. PCA analyses organized varieties at moderate N application (N60, 60 kg N/ha) into four groups represented by green (N-use efficient), pink (moderately N-use efficient), brown (moderately N-use inefficient) and blue (N-use inefficient) based on mean RSI, RNDVI and NAE.

4.4.2. Hierarchical agglomerative cluster analysis (HACA) for PCA validation

HACA was performed on three agro-physiological parameters, including RSI, RNDVI and NAE at an optimum N application rate (N60) to categorize wheat varieties on the basis of their response to nitrogen regimes into four clusters (Figure 4.2). Cluster 1 (FSD-08, PIRSBK-08, NARC-09 and T-8), cluster 2 (TD-1, PAKISTAN-13, AAS-11, CHAKWAL-50), cluster 3 (INQILAB-91 and GA-2002) and cluster 4 (SH-2002 and AARI-1) were categorized as N-use efficient, moderately N-use efficient, moderately N-use inefficient and N-use inefficient varieties, respectively. The classification of 12 wheat varieties at N60 through PCA into four

groups, represented by the same colors in both the PCA plot and dendrogram, was found in complete agreement with each other.

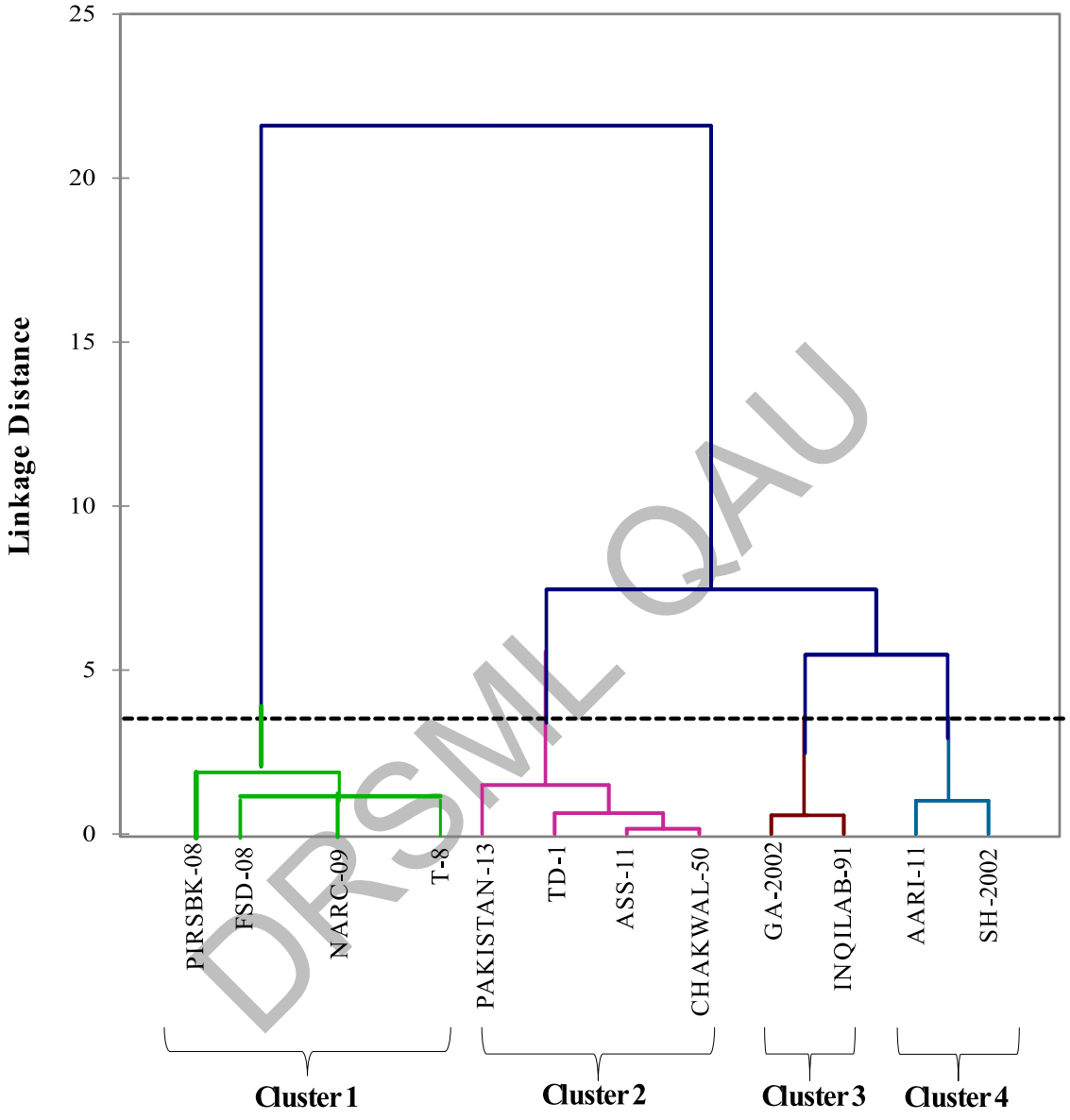

Figure 4.2. Dendrogram analysis showing four clusters, i.e., cluster 1 (N-use efficient), cluster 2 (moderately N-use efficient), cluster 3 (moderately N-use inefficient) and cluster 4 (N-use inefficient).

4.4.3. Canopy temperature depression under varied nitrogen levels

The CTD increases with elevating N levels ultimately helped wheat varieties to lower canopy temperature to better cope with terminal heat stress. In the present study, among different N application rates, mean CTD was lower, i.e., 3.45 ◦C at N0 (0 kgN/ha) followed by 4.86 and 5.44 ◦C at N60 (60 kgN/ha) and N120 (120 kgN/ha), respectively, on an average of

two years of field data of CTD for the studied varieties (Figure 4.3). N-use efficient varieties (FSD-08, PIRSBK-08, NARC-09 and T-8) along with one moderately N-use efficient variety, i.e., T-8, showed significant increases in CTD value with increasing N levels as compared to other studied varieties (Figure 4.3).

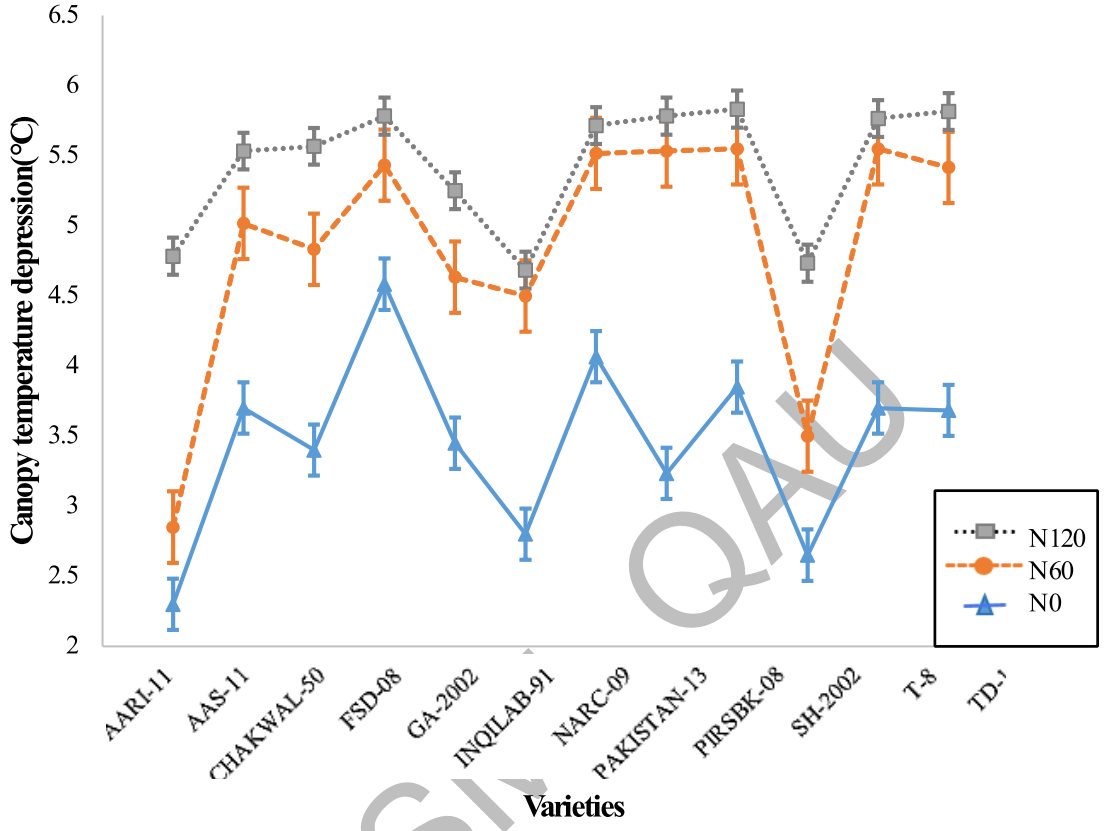

Figure 4.3. Comparison of mean canopy temperature depression (◦C) of twelve wheat varieties cultivated under three different N levels (units?) ***for two years at the National Agricultural Research Centre.***

4.4.4. Agro-physiological traits

4.4.4.1. Plant height (PH)

The data of PH in Appendix 4.1 revealed that PH of the crop was affected by N levels during both cropping years. Escalation in the N application rate increased PH significantly, as mean PH was 96.21 cm at N120, 94.25 cm at N60 and 91.90 cm at N0 from two years of average data. Different varieties have also shown significant variations for PH in both years. A significant increase in PH was observed for FSD-08 (112.42 cm) as compared to other varieties, while minimum PH was measured for CHAKWAL-50 (72.92 cm) in the first cropping season, and for GA-2002 (76.51 cm) in the second cropping season. Mean PH ranged from 79.49 to 112.42 cm among different varieties (Appendix 4.1).

4.4.4.2. Tiller per plant (TpP)

From Appendix 4.1, it is evident that mean tiller per plant (TpP) showed non-significant variation with increase in the N application rate, i.e., 4.66, 4.16 and 3.88 at N120, N60 and N0, respectively, during the first year (2016–2017), and showed a reduced level of significance in the second year (2017–2018). Statistically significant variations have been shown by other varieties. This variation might be caused by better response of wheat varieties to the nitrogen application rate at the tillering stage, which ultimately simulates vegetative growth. At low N levels, the tillering bud remains dormant, which was evident from the data trends in this study. In both cropping seasons, FSD-08 exhibited maximum TpP versus other varieties, while minimum TpP was recorded for AARI-11 in both years (Appendix 4.1).

4.4.4.3. Relative SPAD index (RSI)

Statistically significant variation was computed at different N levels, with mean values of 0.88 and 0.78 for N60 and N0, respectively, averaged from the two years of data. The relative SPAD values of wheat varieties at different N levels are illustrated in Appendix 4.1. The mean RSI values drastically increased from 0.50 to 0.99 in different wheat varieties. The highest mean RSI value was pragmatic in FSD-08, which is same (0.99) in both cropping seasons (Appendix 4.1), while the minimum mean RSI value averaged from the two years of data was detected in AARI-11.

4.4.4.4. Canopy temperature depression (CTD)

The CTD value was high at N120 in both cropping seasons. Mean values for CTD at N120, N60 and N0 were 5.44, 4.86 and 3.45 ◦C, respectively. Varietal response was statistically significant for CTD measurements. Mean CTD values from the two years average data ranged from 3.31 to 5.27 ◦C among different varieties. FSD-08 showed the maximum CTD, which was 5.22 ◦C in 2016–2017 and 5.31 ◦C in 2017–2018. Minimum CTD was calculated for AARI-11 with a mean value of 3.31 ◦C averaged from two years of data (Appendix 4.1).

4.4.4.5. Nitrogen agronomic efficiency (NAE)

The mean NAE values averaged from the two years of data were highest in SH2002 (7.95 kg/kg) followed by PAKISTAN-13 (6.50 kg/kg), as both of these varieties were considered as nitrogen inefficient due to more reduction in grain yield at N0 (no fertilization) and at N60 and N120 (optimum and maximum N fertilization, respectively). FSD-08 showed a minimum mean NAE value of 2.16 Kg/kg from the two years average data due to a reduction in grain yield at N0 (no fertilization) and at N60 and N120 (optimum and maximum N fertilization, respectively as shown in Appendix 4.1.

4.4.4.6. Relative normalized difference vegetation index (RNDVI)

Statistically significant increases in RNDVI with a cumulative amount of N fertilizer were evident from Appendix 4.1, and this trend was significant in both cropping seasons, as mean values of RNDVI were 0.96 and 0.75 at N60 and N0, respectively. The significance level among varieties differed greatly for RNDVI at different N application rates. FSD-08 showed a maximum mean RNDVI value of 1.03; however, AARI-11 showed a minimum mean RNDVI value averaged from the two years of data of 0.56 (Appendix 4.1).

4.4.5. Yield-related traits

4.4.5.1. Grains per spike (GpS)

GpS increased significantly with increases in N levels, as mean GpS from the two years average data were recorded as 52.44 at N120, 48.40 at N60 and 43.69 at N0, and this trend was linear for both years (Appendix 4.2). FSD-08 produced the maximum number of GpS, i.e., 64.11 (2016–2017) and 65.22 (2017–2018) as compared to other varieties. Minimum GpS was produced by AARI-11 in both years with a mean value of 38.22 (Appendix 4.2).

4.4.5.2. Spike length (SL)

The differences in SL at different N levels were significant in both cropping seasons (Appendix 4.2). A significant increase in mean SL from the average of the two years of data was observed, i.e., 9.66 cm at N120 followed by 8.88 cm (N60) and 8.48 cm (N0). The studied varieties exhibited highly significant variations for SL in both years. FSD-08 showed maximum SL in both years, i.e., 11.78 cm (2016–2017) and 11.51 cm (2017–2018). T-8 and TD-1 were at par statistically with SL of 9.37 and 9.31 cm during the first cropping season, whereas a minimum SL of 7.11 and 7.32 cm was recorded for SH-2002 in both years. Mean values of SL (averaged from the two years data) ranged from 7.22 to 11.51 cm.

4.4.5.3. Thousand kernel weight (TKW)

The data of Appendix 4.2 affirmed a linear and significant increase in TKW with increases in the N application rate in both years. Mean TKW from the average of two years of data was 48.57, 44.57 and 41.24 g at N120, N60 and N0, respectively. Selected varieties showed highly significant variations for TKW in 2017–2018, while less significant variations in 2017–2018 were shown for varieties regarding N level interaction. Maximum TKW was recorded for FSD-08, i.e., 48.34 g in 2016–2017 and 48.61 g in 2017–2018. However, differences between AAS-11, PAKISTAN-13 and CHAKWAL-50 were statistically

equivalent during the second cropping season, while minimum mean TKW was shown for AARI-11, i.e., 32.87 g from the two years average data.

4.4.5.4. Biological yield (BY)

The BY was affected by N levels. The highest mean of BY from the average two years of data was calculated at N120, i.e., 11,117 kg/ha followed by N60 (10,711 kg/ha) and N0 (10,202 kg/ha). Nitrogen fertilization significantly impacted BY in both cropping seasons. Statistically significant variation was determined among different varieties in both cropping years. Maximum BY was recorded for FSD-08, i.e., 12,564 kg/ha in 2016–2017 and 13,096 kg/ha in 2017–2018. Minimum BY was chronicled for AARI-11, which is 7389 and 7383.50 kg/ha in the first cropping and second cropping season, respectively (Appendix 4.2).

4.4.5.5. Grain yield (GY)

Mean GY values averaged from two years of data as 3134.15 at N120, 2662.75 at N60 and 2430.05 at N0, recorded in kg/ha, were significant (Appendix 4.2). The studied varieties exhibited highly significant variations for GY in both years. FSD-08, PIRSBK-08 and NARC-09 yielded high amounts with mean values of 3819.10, 3693.90 and 3667.90 kg/ha, respectively, as compared to other varieties in both cropping seasons. However, minimum GY was recorded for INQILAB-91, i.e., 1642.4 kg/ha in the first season and 1655 kg/ha in the second season. Mean GY from two-year averaged data ranged from 1648.70 to 3819.10 kg/ha (Appendix 4.2).

4.4.5.6. Harvest index (HI)

The effects of different N levels on harvest index (HI) are presented in Appendix 4.2. HI showed a significant increasing trend due to increases in the N application rate. The highest mean HI was observed at N120 (28.10%) followed by N60 (24.39%) and N0 (23.13%) from two-year averaged data. The varieties displayed significant variations for HI in both years. The highest mean for HI was calculated for T-8 (30.81%), followed by TD-1 (30.98%). The differences in HI between FSD-08, PIRSBK-08 and NARC-09 were statistically similar in both years, with less difference. In addition, minimum mean HI was recorded for INQILAB-91 and AARI-11, as both showed the same mean value of 18.51% from two-year averaged data. Mean HI values from two-year averaged data ranged 18.51 to 30.98% among studied varieties (Appendix 4.2).

4.4.6. Relationship between RSI and RNDVI

A strong association was found between RSI and RNDVI (R^2 = 0.8062) at different N levels (Figure 4.4). Highly N-use efficient and N-use inefficient varieties exhibited deviation from the trend line, which is presented by square boxes in Figure 4.4. Varieties revealing low RSI and RNDVI value are at the start and below the trend line (enclosed square boxes in Figure 4.4). These deviations corresponded to SH-2002 and AARI-11, which are N-use inefficient varieties, whereas N-use efficient varieties such as FSD-08, PIRSBK-08, NARC-09 and T-8 are above the trend line, showing high RSI and RNDVI values (enclosed square boxes in Figure 4.4). Thus, the results in Figure 4.4 were verified and are in complete agreement with the findings of the PCA plot (Figure 4.1), HACA (Figure 4.2) and means of Appendix 4.2 and 4.3. However, the rest of the varieties including TD-1, PAKISTAN-13, AAS-11, CHAKWAL-50 (moderately N-use efficient) and INQILAB-91, GA-2002 (moderately N-use inefficient) are near the trend line. A similar trend of high and low RSI and RNDVI values was observed in this study, depicting that any variety that has an RSI value must have a high RNDVI value and vice versa. Thus, hereafter, the relationship of both RSI and RNVI with other phenotypic traits is evaluated simultaneously as RSI on the primary Y-axis and RNDVI on the secondary Y-axis.

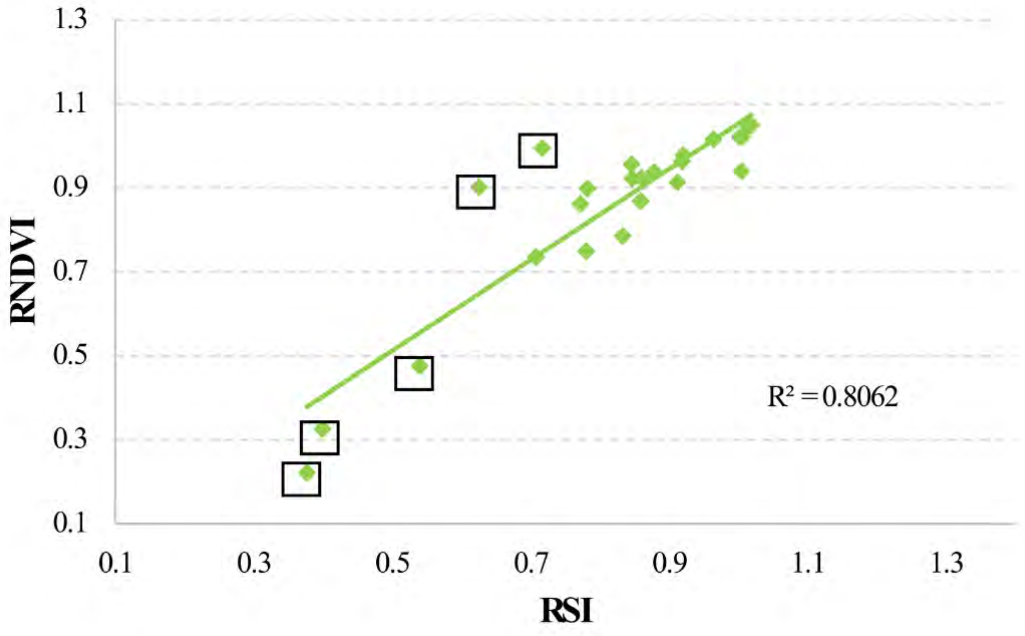

Figure 4.4. The relationship between relative SPAD index (RSI) and relative normalized difference vegetation index (RNDVI) of 12 wheat varieties cultivated under three N levels. The points marked with a square box show deviation of the varieties from the regression trend line.

4.4.7. Relationship of RSI and RNDVI with NAE

In this study, an inverse relationship was observed for RSI and RNDVI with NAE (Figure 4.5). NAE was calculated by subtracting grain yield at no nitrogen fertilization (N0) from the yield of the N treatments, i.e., N60 and N120. Thus, wheat varieties with a low NAE value will have a high RSI and RNDVI value and will be categorized as nitrogen-use efficient. A similar trend was observed in Figure 4.5, which demonstrated a downward trend line, while N-use efficient varieties such as FSD-08, PIRSBK-08 and NARC-09 lie above the trend line, having low mean NAE values but high mean RSI and RNDVI values and vice versa for SH-2002 and AARI-11, which are N-use inefficient varieties. The rest of the varieties demonstrated moderate variations and were closer to the trend line, being moderately N-use efficient and inefficient and having low regression values, i.e., R^2 = 0.0071 and R^2 = 0.0922 to depict moderately significant relationships between RNDVI/NAE and RSI/NAE.

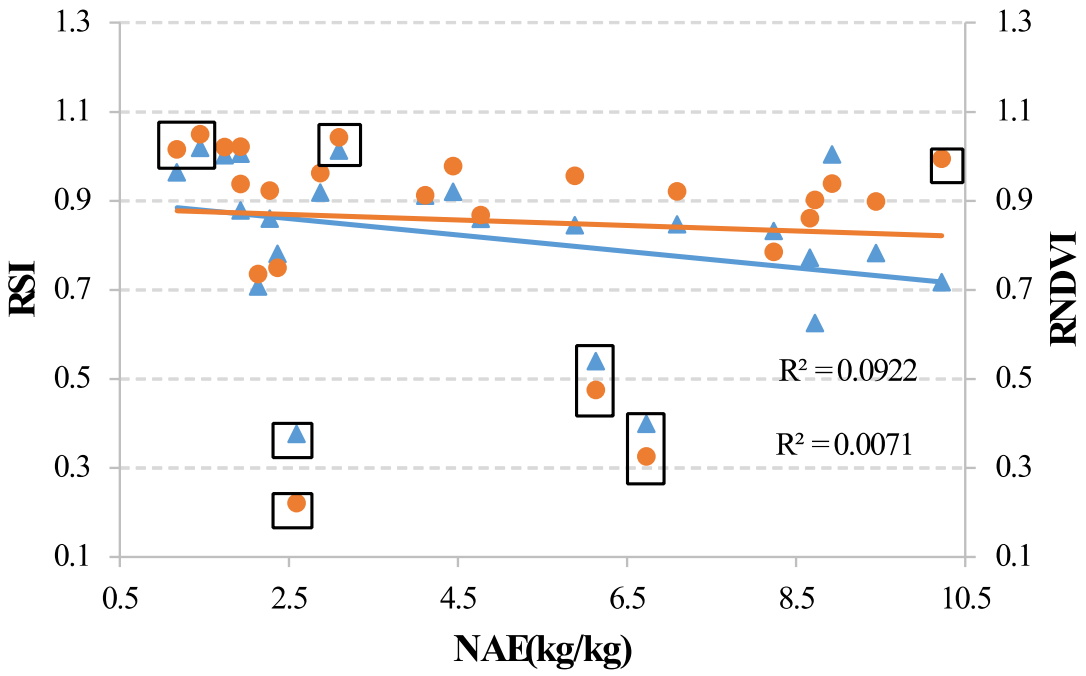

Figure 4.5. The relationship of relative SPAD index (RSI) and relative normalized difference vegetation index (RNDVI) with nitrogen agronomic efficiency (NAE) of 12 wheat varieties cultivated under three N levels. The points marked with a square box show deviation of the varieties from the regression trend line.

4.4.8. Relationship of RSI, RNDVI with yield and yield-related traits

This study evaluated N-use efficiency by measuring N concentration in each variety at the anthesis in the form of RSI and RNDVI, indicating that these traits have a significant

relationship with N supply and NUE. Figure 4.6 demonstrates that grain yield and yield components increased by escalating the N concentration in leaves as a consequence of more N fertilization. In the present study, RSI and RNDVI have linear relationships with applied N fertilization, with GY at $R^2 = 0.72$ and $R^2 = 0.48$, respectively (Figure 4.6A). A significant linear correlation was observed for RSI and RNDVI with BY at $R^2 = 0.78$ and $R^2 = 0.56$, respectively (Figure 4.6B). Varieties with high RSI and RNDVI values, i.e., FSD-08, PIRSBK-08, NARC-09 and T-8, also showed high GY and BY (Figure 4.6A, 4.6B and Appendix 4.2). There was significant association of RSI and RNDVI with PH at $R^2 = 0.39$ and $R^2 = 0.31$, respectively (Figure 4.6C). Both RSI and RNDVI displayed significant association with HI ($R^2 = 0.64$ and $R^2 = 0.41$, respectively) across the various N levels (Figure 4.6D). There were strong significant effects of RSI and RNDVI values on GpS with $R^2 = 0.59$ and $R^2 = 0.44$, respectively (Figure 4.6E). The relationship of RSI and RNDVI was positive and linear regarding SL ($R^2 = 0.75$ and $R^2 = 0.49$, respectively) (Figure 4.6F). The regression of TpP with RSI and RNDVI was linear and positive, with $R^2 = 0.64$ and $R^2 = 0.43$, respectively (Figure 4.6G). There was a positive exponential relationship with TKW regarding RSI and RNDVI (Figure 4.6H), with $R^2 = 0.82$ and $R^2 = 0.61$, respectively. A strong association was found between CTD and that of RSI and RNDVI, with regression values of 0.68 and 0.55, respectively (Figure 4.6I). The temperature was critical for all growth stages of the wheat crop. Hence, maintaining elevated NDVI under high temperature stress, such as terminal heat during grain filling, can be considered a sign of stress tolerance with potential use in wheat germplasm screening. High-yield wheat cultivars maintained higher NDVI values, whereas low-yield cultivars expressed a steep descent. Multiple linear regression was calculated to show the relationship of RSI and RNDVI with the agro-physiological traits of 12 wheat varieties grown under three N levels (Appendix 4.3).

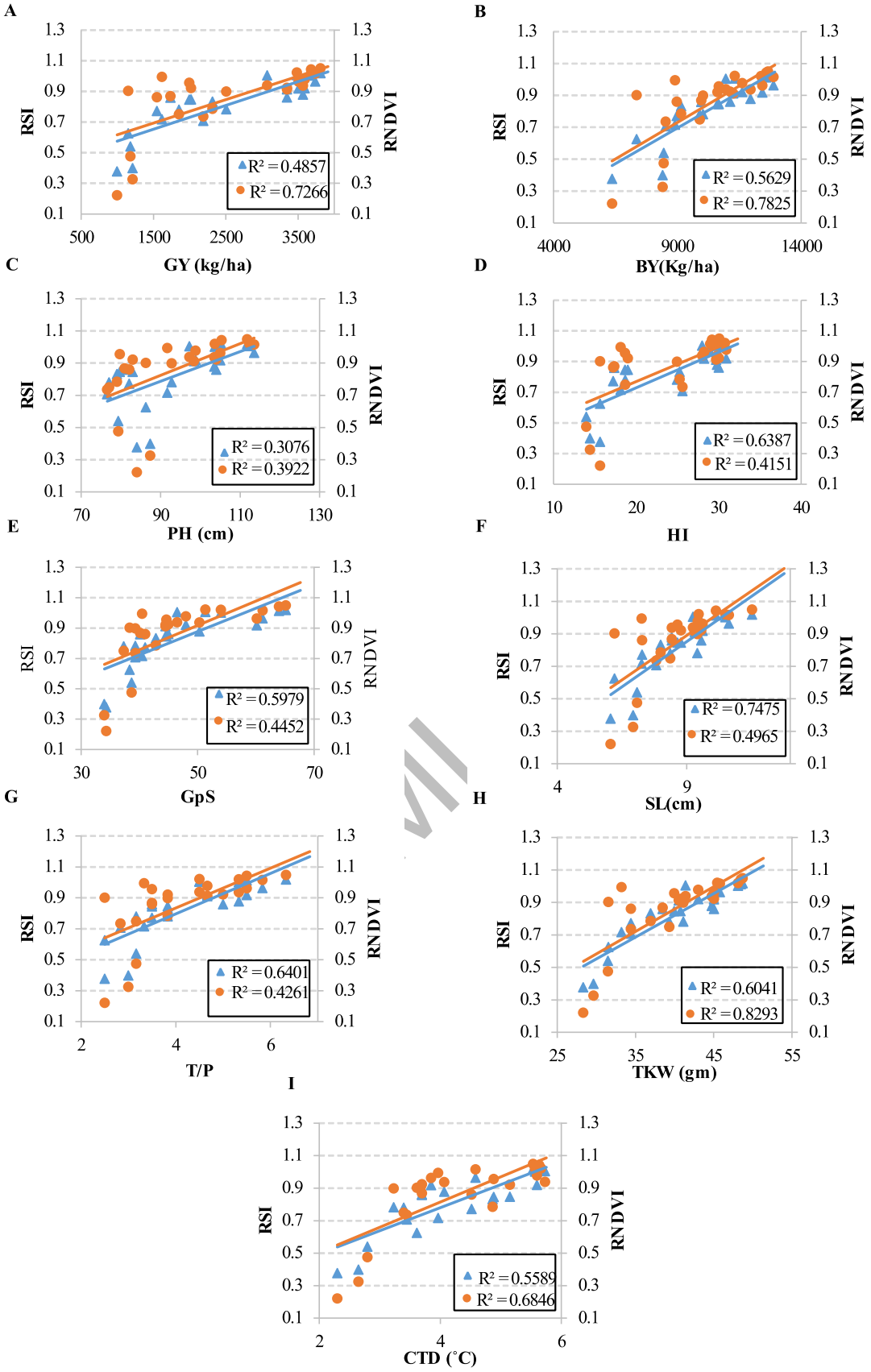

Figure 4.6. The relationship of RSI and RNDVI with agro-physiological traits of 12 wheat varieties grown under three N levels: (A) grain yield (GY), (B) biological yield (BY), (C) plant height (PH), (D) harvest index (HI), (E) grains per spike (GpS), (F) spike length (SL), (G)

tiller per plant (TpP), (H) thousand kernel weight (TKW), (I) canopy temperature depression (CTD), ▲ ; RSI, ●; RNDVI.

4.5. Discussion

Nitrogen supply has a direct impact on a crop's vigour and results in more grain yield; thus, N fertilization in wheat contributes to enhanced yield as observed in the present work under variable nitrogen and terminal heat stress conditions, which have been previously reported (Adnan et al., 2016). The results depict significant variations between N levels and varieties for RSI, RNDVI, CTD, NAE, GY, BY, and HI, along with other yield-related traits.

Developing N-use efficient wheat varieties has been a challenge for wheat breeders (Cormier et al., 2016; Prey et al., 2019). Several genes are involved that control grain yield under varied N levels, with effects of not only the genetic backgrounds but also of the environments having been reported (Mahjourimajd et al., 2016). To determine the most desirable wheat varieties, RSI and RNDVI provided more effective assessments (Islam et al., 2014; Sultana et al., 2014). Identifying wheat varieties that are more responsive to N levels and utilizing them efficiently can decrease the N fertilizer application rate, which is annually lost due to leaching into the soil and water ways. This ultimately reduced not only fertilizer input costs but also the amount of nutrient losses. It also increased crop yields (Rosenstock et al., 2013). In the present research work, phenotyping was performed by using precision agricultural approaches to N response-related factors, specifically RNDVI and RSI, which determined the significant positive correlation with nitrogen-use efficiency (NUE), nitrogen nutrition index (NNI) and GY (Debaeke et al., 2006; Sultana et al., 2014). Multivariate analytical techniques, i.e., PCA and HACA, can categorize wheat varieties in terms of N-use efficiency and inefficiency with precision and accuracy. Similar verification methodologies were used previously (Ali et al., 2018; Wolf & Kirschner, 2013). Based on these two multivariate techniques, we recommend cultivation of N-use efficient varieties, i.e., FSD-08, PIRSBK-08, NARC-09 and T-8 (cluster 1), in rain-fed areas of Pakistan, which should receive preference over that of moderately N-use efficient and inefficient varieties on account of their better response to optimum N fertilization regimes (Figure 4.1). Wheat varieties that were best adapted to a particular area and that can better exploit available resources should be preferred over other varieties cultivated in that particular area (Rehman et al., 2015). These findings were in agreement with already reported results by (Gaju et al., 2016; Rosenstock et al., 2013), in which the SPAD index (Appendix 4.1) and normalized difference vegetation index (NDVI)

accurately predicted grain yield of wheat crop at the anthesis stage when nitrogen (N) was a limiting factor.

CTD provides a more accurate assessment, as it is calculated as the difference between crop canopy temperatures and the ambient temperature (Rosyara et al., 2008). The present study indicated that a higher N dose resulted in a better thermal environment of the crop canopy. These findings are in line with already reported data that an increase in N application rate results in a decrease in CT values (Elfadil et al., 2012; Ward, 2015). Similar results have already been reported, in which an increase in N fertilization application decreases CT (canopy temperature) in wheat crop by 1.0–2.0 ˚C (Lepekhov, 2022).

Our results showed that N fertilization had a significant impact on all agro-physiological and yield-related traits. PH generally increased with increases in the N application rate due to elongation of the nodes in the wheat crop (Mosanaei et al., 2017). Differences in genetic makeup of different varieties is one of the main attributes responsible for variation in PH. The results of the present study were in line with the reported data that higher levels of N significantly improved plant height, as more available nitrogen is responsible for this increase (Mattas et al., 2011; Sultana et al., 2013). An appropriate amount of nitrogen application can regulate tiller number (Yang et al., 2019). Productive tillers are the primary determinant of grain yield. Moreover, external factors such as N application rate and genetic attributes contribute to tillering capacity of any genotype. In the present study, a higher N fertilizer application resulted in greater SPAD values. These findings are in line with previously reported results (Islam et al., 2014). The RSI value becomes elevated with an increase in N application rate (Debaeke et al., 2006). This trend was also verified previously, in which SPAD readings have a direct correlation with leaf chlorophyll content at the anthesis stage in wheat (Arregui et al., 2006). It was observed that NAE also shows a significant correlation with N application rates. More N fertilization resulted in higher RNDVI values in this study. A similar trend of an increase in NDVI value with increasing N levels was also reported in past scientific studies (Sultana et al., 2014). These results are also in line with already reported outcomes that the NDVI has a positive correlation with the biomass and amount of N accumulated in aerial plant parts (Vian et al., 2018b). Furthermore, plant density is one of the imperative factors that determines the yield in wheat crop, which can be efficiently measured with NDVI values.

The GpS increased with an increase in N application rate. A similar trend was reported that the effect of N levels on GpS was also statistically significant among different varieties (Mandic et al., 2015). GpS (grains per spike) has been used to determine the yield potential of

a wheat variety (Afridi et al., 2010; Guarda et al., 2004). SL improved with an increase in N fertilization rate. Longer spikes have ensured higher grain yield in wheat crops (Khalil et al., 2011). The increase in N rate significantly affected TKW. This result coincides with a previous study on the effect of N levels on TKW (Linina & Ruza, 2018). TKW is an important component of grain yield, as maximum TKW was obtained from wheat varieties that were sown at the highest N application rate (Hussain et al., 2002). BY is an important representative of plant overall growth and performance, as it is one of the most essential yield parameters. A trend between an increase in BY and an increase in N application rate was reported by Adnan et al., (2016). These results are in line with many previous reports on the impact of N fertilization on wheat (Guarda et al., 2004; Hussain et al., 2006; Maqsood et al., 2002a). A significant increase in GY of staple crops, including wheat, is in dire need at present. The increase in N application rate significantly increases grain yield by improving different yield components, including spike length, grain per spike and thousand kernel weight (Cantu et al., 2011). An increasing trend in HI by elevating N levels was observed. HI is directly related with above-ground total dry matter (TDM) and is impacted by genotype and environment interaction (Maqsood et al., 2014).

Nitrogen response in the form of nitrogen-use efficiency (NUE), nitrogen nutrition index (NNI) and grain protein content (GPC) shows an inverse correlation with leaf chlorophyll content and vegetation index, i.e., NDVI (D. Arnall et al., 2006; Mandic et al., 2015; Nguyen et al., 2016). Moreover, CTD, BY and HI have a strong association with RSI and RNDVI. This confirmed the findings of many previous studies (Mandic et al., 2015; Ward, 2015). For wheat, the NDVI values were significantly correlated with the GY with R^2 value, ranging from 0.601 to 0.809 for the reproductive to early ripening stages, which was reported by many studies previously (Ali et al., 2017; Fiez et al., 1995; Prost & Jeuffroy, 2007; Yang et al., 2018b). Varieties with high RSI and RNDVI values also produce higher biological and grain yield. These results are in line with previously reported findings (Mansour et al., 2017). Significant correlations of $R^2 = 0.71$ were obtained between particular hyperspectral NDVI indices and all yield traits of wheat at the medium milk stage, which verified the results of the present study (Cabrera-Bosquet et al., 2011).

4.6. Conclusions

Effective nitrogen (N) fertilizer application is essential for attaining high wheat production. Identifying varieties that can utilize applied N more efficiently is a potential way

of reducing N losses through leaching and denitrification. Therefore, the efficient use of nitrogen is urgently needed. The findings of this study indicate that an increase in N fertilizer application results in better crop canopies with lower temperature ranges (as observed from the significant increase in mean CTD value from 3.45 at N0 to 5.47 at N120), as well as higher chlorophyll content (RSI) and vegetation index (RNDVI) under the heat-stressed conditions of Pakistan. Based on the findings of the present study, 60 kg N/ha is recommended for achieving higher yields from N-use efficient varieties (FSD-08, PIRSBK-08, NARC-09 and T-8), but it is not a sufficient dose for the rest of the varieties for attaining maximum yield in rain-fed conditions of Pakistan. FSD-08 was recorded to be the best variety compared to the other varieties, followed by PIRSBK-08, NARC-09 and T-8, which can be grown for economic yields, whereas SH-2002 and AARI-11 are N-use inefficient varieties with minimum mean GY productions of 1761 and 1398.7 kg/ha, respectively. However, the varietal response in utilizing N fertilizer in canopy cooling and the accumulation of more N fertilizer was reflected in the form of RSI, RNDVI and NAE in the present study. These parameters reveal that N fertilizer application should be delivered according to the efficiency and response of each variety. Moreover, this study also concluded that multivariate analytical techniques, i.e., PCA and HACA, can categorize wheat varieties in terms of N-use efficiency and inefficiency with precision and accuracy. The development of nutrient-use-efficient and heat-stress-tolerant wheat varieties using conventional and modern breeding approaches is promising. The current findings can be used to investigate the role of nitrogen fertilizer in lowering crop canopy temperature at the molecular level. In the last decade, many omics approaches have transformed research strategies that plant biotechnologists and breeders have used to investigate underlying abiotic stress tolerance mechanisms. There is a dire need for a deeper understanding of nutrient-use and heat-stress-tolerance mechanisms of different wheat varieties at the transcriptomic level. The use of genomics, proteomics, metabolomics, and transcriptomics data sets are needed rather than relying on phenomics data sets only.

Chapter#5

Wheat NAM genes regulate the majority of early monocarpic senescence transcriptional changes including nitrogen remobilisation genes

Chapter # 5

Wheat *NAM* Genes Regulate the Majority of Early Monocarpic Senescence Transcriptional Changes Including Nitrogen Remobilisation Genes

5.1. Abstract

Senescence enables the remobilisation of nitrogen and micronutrients from vegetative tissues of wheat (*Triticum aestivum* L.) into the grain. Understanding the molecular players in this process will enable the breeding of wheat lines with tailored grain nutrient content. The *NAC* transcription factor *NAM-B1* is associated with earlier senescence and higher levels of grain protein, iron, and zinc content due to increased nutrient remobilisation. To investigate how related *NAM* genes control nitrogen remobilization at the molecular level, we carried out a comparative transcriptomic study at seven time points (3, 7, 10, 13, 15, 19 and 26 days after anthesis) in wild type and *NAM* RNA interference (RNAi) lines with reduced *NAM* gene expression. Approximately 2.5 times more genes were differentially expressed in WT than *NAM* RNAi during this early senescence time course (6,508 vs 2,605 genes). In both genotypes, differentially expressed genes were enriched for GO terms related to photosynthesis, hormones, amino acid transport and nitrogen metabolism. However, nitrogen metabolism genes including *glutamine synthetase* (*GS1* and *GS2*), *glutamate decarboxylase* (*GAD*), *glutamate dehydrogenase* (*GDH*) and *asparagine synthetase* (*ASN1*) showed stronger or earlier differential expression in WT than in *NAM* RNAi plants, consistent with higher nitrogen remobilisation. The use of time course data identified the dynamics of *NAM*-regulated and *NAM*-independent gene expression changes during senescence, and provides an entry point to functionally characterise the pathways regulating senescence and nutrient remobilisation in wheat.

5.2. Introduction

Wheat supplies approximately 20 percent of calories in the human diet and is an important source of protein and micronutrients. Beyond nutritional benefits, wheat grains with higher protein content are associated with increased bread making quality and attract a price premium. Although nitrogen (N) fertilization is commonly used to increase grain protein content, high nitrogen fertilization leads to higher production costs and environmental pollution (Aranguren et al., 2021; Martínez-Dalmau et al., 2021). Alternatively, genetic

approaches can be used to increase protein content, although identifying the genetic loci to target remains a challenge.

The final grain yield and nutrient content depends on the accumulation and transport of carbon, nitrogen and other nutrients from the vegetative tissues to the developing grain. The remobilisation of nutrients is strongly influenced by the process of senescence, which is a developmentally regulated programme to remobilise nutrients from vegetative tissues to the developing grain. The starting time and progression of flag leaf senescence influences the remobilisation of nutrients and the final yield (Distelfeld et al., 2014), with the flag leaf contributing a significant proportion of nitrogen to the seed by degrading and recycling proteins (Bogard et al., 2010; Havé et al., 2017; Kichey et al., 2007). Delayed leaf senescence can be associated with prolonged photosynthesis and increased grain yield but also decrease grain protein content due to reduced nutrient remobilisation from the leaf tissues (Alpuerto et al., 2021; Uauy et al., 2006). Therefore, altering the rate and progress of senescence can influence final yield and protein content of wheat grain. Understanding the molecular components influencing flag leaf senescence and nitrogen remobilization can help to improve nitrogen remobilisation efficiency and grain protein content in wheat.

The identification of the *NAM-B1* gene which is a NAC transcription factor that influences senescence and grain nutrient content opens the door to identify the molecular pathways regulating senescence and nutrient remobilisation in wheat. *NAM-B1* was identified through positional cloning as the causal gene for *Gpc-B1* which affects grain protein content (Uauy et al., 2006). *NAM-B1*, together with its homoeologs *NAM-A1* and *NAM-D1*, influences senescence and enhance nutrient remobilisation (Avni et al., 2014; Cormier et al., 2015; Harrington et al., 2020). Most modern wheat cultivars carry a non-functional allele of *NAMB1*, whereas the functional allele, which was identified through map-based cloning, is mainly found in wild emmer wheat and landraces (Hagenblad et al., 2012). Closely related paralogs of *NAM-B1* have been identified on chromosome 2 which also regulate senescence and nutrient remobilisation (*NAM-A2*, *NAM-B2* and *NAM-D2*) (Borrill et al., 2019; Pearce et al., 2014). A study of *NAM* RNAi lines with reduced expression of the *NAM-B1* homoeologs and paralogs showed that remobilisation of micronutrients and nitrogen was strongly reduced in the *NAM* RNAi lines, which directly implicates *NAM* genes in the control of nutrient remobilisation during senescence (Waters et al., 2009). These *NAM* genes provide a valuable entry point to decipher the control of monocarpic senescence and nitrogen remobilization in wheat at the molecular level.

A transcriptomic study of the same *NAM* RNAi lines at 12 days after anthesis revealed that *NAM* genes regulate transporters, hormone regulated genes and transcription factors at this early stage of senescence in flag leaves (Cantu et al., 2011). Additional *NAM*-regulated genes in flag leaves were identified by comparing wild type plants to lines mutated in either *NAMA1* or *NAM-A1* and *NAM-B2* at 0, 12 and 22 days after anthesis (Pearce et al., 2014). Consistent with Cantu et al. (2011), *NAM*-regulated genes included photosynthesis-related genes and many zinc and iron transport genes. These studies provide a valuable insight into the transcriptional effects of *NAM* genes but the small number of time points limits our ability to understand the influence of *NAM* genes throughout monocarpic senescence. Furthermore, reduced sequencing costs and advances in genome assemblies and annotation for wheat allow more accurate analysis than was possible when previous studies on *NAM*-regulated genes were carried out using *de novo* transcriptome assemblies (Cantu et al., 2011) or earlier genome assemblies (Harrington et al., 2020; Pearce et al., 2014). Studies using time course data can reveal the dynamics of gene expression during a developmental process. Previous studies have characterised changes in flag leaves at the transcriptome level during senescence in wheat (Borrill et al., 2019; Zhang et al., 2018), but we do not have a full understanding of the timing of gene expression controlled by *NAM* genes for nutrient remobilization during monocarpic senescence.

To address the lack of time-resolved understanding of *NAM* gene regulation of senescence and nutrient remobilisation, we analysed flag leaf tissues at seven time points from wild type and *NAM* RNAi wheat plants. Previous work demonstrated that *NAM* genes strongly influence nitrogen remobilisation but the downstream molecular pathways were largely unknown. Therefore, we characterised gene expression changes in nitrogen-associated genes during senescence in wild type and *NAM* RNAi plants and identified genes through which *NAM* genes may influence nitrogen remobilisation. These putative *NAM* gene targets may represent target genes to improve nitrogen remobilisation in wheat.

5.3. Methods

5.3.1. Plant material and growth conditions

Wild type wheat (*Triticum aestivum*) plants cv. Bobwhite and sibling lines with reduced levels of *NAM* gene expression (*NAM* RNAi) were generated by Uauy et al. (2006). All plants were grown as previous described in Borrill et al. (2019) and the samples analysed

in this manuscript for the wild type are a subset of those previously published in Borrill et al. (2019).

Briefly, we pre-germinated WT and *NAM* RNAi seeds on Whatman filter paper for 48 hours at 4°C, followed by 48 h at ~20°C. These germinated seeds were then sown in trays (P40) containing a mixture of horticultural grit (15%) and fine peat (85%). We transferred individual plants to 1L square pots containing Petersfield Cereal Mix at 2 to 3 leaf stage. Plants were grown in light (16h) and dark (8h) at the temperature of 20°C and 15°C respectively. We tagged the main tiller in each pot for anthesis date, phenotyping and sample collection.

5.3.2. Phenotypic data collection

We used SPAD-502 chlorophyll meter (Konica Minolta) to measure the flag leaf chlorophyll content at seven-time points (3, 7, 10, 13, 15, 19 and 26 days after anthesis (DAA)). At each time point, we recorded chlorophyll content from five independent plants, measuring eight locations along each flag leaf length, using only the tagged main tiller. Three out of five leaves measured for chlorophyll content were subsequently harvested for RNA extraction.

We measured grain moisture content at the same seven-time points (3, 7, 10, 13, 15, 19 and 26 DAA) at which we measured leaf chlorophyll content. From 5 independent plants, we harvested eight grains from the central spikelets (floret positions 1 and 2) from the tagged spike at each time point. We weighed fresh grains, then reweighed them after drying at 65°C for 72 hours to obtain dry weight. We calculated the percent grain moisture content from the difference in fresh and dry weight of a seed.

5.3.3. Sample collection

For RNA extraction, we harvested the flag leaf from the tagged main tiller at seven time points: 3, 7, 10, 13, 15, 19, and 26 days after anthesis (DAA) for both WT and RNAi lines. From each flag leaf we harvested the middle 3cm lengthways, to focus on a developmentally synchronised section of tissue. Three independent replicates were harvested for each timepoint and genotype. The samples were flash frozen in liquid nitrogen and stored at -80°C.

5.3.4. RNA extraction

We ground the leaf samples to a fine powder using mortar and pestles pre-chilled with liquid nitrogen. RNA was extracted using Trizol by following the manufacturer's (ThermoFisher) protocol, with 1ml Trizol added to 100mg ground samples. Genomic DNA contamination was removed by using DNAsel (Qiagen) and samples were further cleaned through RNeasy Minikit by following instructions of the manufacturer (Qiagen).

5.3.5. Library preparation and sequencing

Library preparation and sequencing was carried out using the same methods as described in Borrill et al., (2019). Briefly after RNA quality confirmation, the TruSeq RNA protocol v2 was used for the construction of TruSeq RNA libraries on PerkinElmer Sciclone (Illumina 15026495 Rev.F). After adaptor ligation, the libraries were size selected using Beckman Coulter XP beads (A63880). The PCR used a primer cocktail which enriched DNA fragments having adaptors at both ends. Library insert sizes were confirmed by running an aliquot of the DNA library on a PerkinElmer GX (PerkinElmer CLS760672) and concentration measured using the Tecan plate reader.

After normalization, the TruSeq RNA libraries were equimolar pooled into two final pools using Qiagen elution buffer (one pool contained WT samples, one pool contained RNAi samples). Each library pool was diluted to a 2nM concentration using sodium hydroxide (NaOH). Five µL of this solution was added to 995µL of HT1 (Illumina) to give a final concentration of 10pM. The diluted library pool (120 µL) was spiked with PhiX control v3 (1% v/v) and transferred to a 200 µL strip tube and placed on ice before loading on the Illumina cBot. The HiSeq PE Cluster Kit v3 was used to cluster the flow cell on the Illumina cBot, using the Illumina PE_Amp_Lin_Block_Hyb_V8.0 protocol. After clustering the flow cell was transferred onto the Illumina HiSeq 2000/2500 instrument. The sequencing chemistry was HiSeq SBS Kit v3 coupled with HiSeq Control Software 2.2.58 and RTA 1.18.64. Reads in bcl format were demultiplexed using the 6bp Illumina index by CASAVA 1.8, allowing for a one base-pair mismatch per library, and converted from FASTQ format by bcl2fastq.

5.3.6. Transcriptome analysis -mapping

We pseudoaligned the samples using Kallisto v0.44.0 with default settings to the RefSeqv1.0 annotation v1.1 (Appels et al., 2018). We noticed that unexpectedly the *NAM*

genes were more highly expressed in the *NAM* RNAi lines than the WT lines. Examining the read alignment we found that the transgenic RNAi construct was mapping to the *NAM* gene transcripts and artificially inflating *NAM* gene expression levels in these samples. To account for this, we substituted these regions of anomalous mapping in each of the *NAM* gene transcripts with Ns (613 to 623 bp, representing on average 29.3% of the transcript length; *TraesCS2A02G201800.1*,*TraesCS2A02G201800.2*,*TraesCS2B02G228900.1*,*TraesCS2B02G228900.2*,*TraesCS2D02G214100.1*,*TraesCS6A02G108300.1*,*TraesCS6A02G108300.2*,*TraesCS6D02G096300.1*,*TraesCS6B02G207500LC.1*,*TraesCS6B02G207500LC.2*). Samples were re-mapped to this masked version of the v1.1 annotation and all subsequent analysis used these re-mapped values. The masked v1.1 annotation is available at https://doi.org/10.6084/m9.figshare.20210774.v1. In total we analysed 42 samples: 3 replicates of 7 timepoints (3, 7, 10, 13, 15, 19 and 26 DAA) for 2 genotypes (WT and *NAM* RNAi). For comparison, the count and TPM (transcripts per million) of all samples were combined into one data frame by using tximport v1.0.3 (Soneson et al., 2015). All scripts used for the data analyses in this manuscript are available at https://github.com/Borrill-Lab/NAM_RNAi_Senescence and input files required to run the scripts can be found at https://doi.org/10.6084/m9.figshare.20210774.v1.

5.3.7. Differential expression analysis

We filtered the data for further analysis to include only high confidence genes; expressed at >0.5 TPM at least in one-time point. This strategy excluded all low confidence gene models and low expressed genes from the data (Ramírez-González et al., 2018). In total 52,395 genes in WT and 52,626 genes in RNAi were expressed at >0.5 TPM. We identified genes that were differentially expressed at each timepoint by comparing WT and RNAi samples using DESeq2 v1.14.1 (Love et al., 2014). We then analysed the data using time-aware differential expression analysis software. The count of expression levels of the genes expressed >0.5 TPM were rounded to the nearest integer to identify differentially expressed genes (DEGs) using ImpulseDE2 v1.10.0 (Fischer et al., 2018). For accuracy, we also identified DEGs through Gradient Tool v1.0 (Breeze et al., 2011) by using the TPM expression level of 52,395 genes in WT and 52,626 genes in RNAi on Cyverse (https://de.cyverse.org/de/) with enabled data normalization option (Merchant et al., 2016). To identify high confidence gene DEGs, we filtered to only consider genes as differentially expressed that were both identified by using ImpulseDE2 at padj <0.001 and Gradient Tool at z-score of > |2|.

5.3.8. Group patterns of differentially expressed genes

We categorized the high confidence DEGs on the basis of the first-time point at which they were either up or down-regulated according to Gradient Tool output for the WT and RNAi time courses separately. The Gradient Tool is based on Gaussian process regression for the identification of gene expression patterns either increasing (up-regulated) or decreasing (down-regulated) at each time point (Breeze et al., 2011). A gene that was first up-regulated at 7 DAA was placed in the "U07" group (up 7 DAA). While a gene that was first downregulated at 7 DAA was categorized in the "D07" group (down 7 DAA). Few genes (~2% of all DEGs) were both up-and down-regulated during either time course (3-26 DAA); these were assigned a group based on their first expression pattern with the opposite trend also indicated. For instance, a gene that showed down-regulation at 7 DAA and then up-regulated at 19 DAA was grouped as "D07U" (the second time point at which differential expression occurred was not reported in the grouping pattern). These grouping patterns for WT and RNAi are available in Supplementary Tables 1 and 2, respectively. The genes with both up and down-regulation trends (~2% of all DEGs) were excluded from further analyses.

5.3.9. GO term enrichment

GO terms were only available for the RefSeqv1.0 annotation, therefore we used the same approach as Borrill et al. (2019) to transfer GO terms to the v1.1 annotation. We only transferred GO terms for genes which were >99% identical across >90% of the sequence (105,182 genes; 97.5% of all HC genes annotated in v1.1). Using GOseq v1.38.0, GO term enrichment was done for each group of DEGs separately (groups were assigned based on first-time point gene expression pattern either increasing (up-regulated) or decreasing (downregulated)).

5.3.10. Nitrogen orthologs identification

We identified a list of genes involved in nitrogen metabolism in Arabidopsis through a literature search (Brumbarova & Ivanov, 2019; Gaudinier et al., 2018; Grallath et al., 2005; Havé et al., 2017; Hirner et al., 2006; Masclaux-Daubresse et al., 2008; Stacey et al., 2006; Su et al., 2004). We then identified their respective orthologs in wheat using EnsemblPlants ortholog information downloaded via BioMart (Kersey et al., 2018). Due to the evolutionary distance between Arabidopsis and wheat it was not possible to assign 1:1 orthologs in many

cases due to within-lineage duplications and gene losses. Therefore, we took an inclusive approach to identifying orthologs, considering that all wheat genes in the gene tree could be orthologs of the associated Arabidopsis gene (Supplementary Table 3). Functional annotation of nitrogen associated genes differentially expressed in WT and RNAi were obtained from literature searches and g:Profiler (Raudvere et al., 2019).

5.4. Results

5.4.1. Phenotypic data and *NAM* gene expression

To examine the transcriptional differences during the initiation of senescence in wild type and plants with reduced *NAM* gene expression (NAM RNAi), we harvested an early time course of flag leaf senescence at 3, 7, 10, 13, 15, 19, and 26 DAA (Figure 5.1A and 5.1B). SPAD chlorophyll meter readings recorded from the flag leaves were similar from 3 to 19 DAA in both WT and RNAi, with a significantly reduced value at 26 DAA in WT compared to RNAi (Figure 5.1C). Grain moisture content decreased significantly between 3 and 26 DAA for both genotypes at a similar rate. By 26 DAA, the grain moisture content (55% in WT and 57% in RNAi) indicated that the wheat plants had reached soft dough stage (GS85) and the time period sampled included the majority of the grain filling period (Figure 5.1D; (Zadoks et al., 1974). We found that as expected, *NAM-A1* and *NAM-D1* were expressed at lower levels in the *NAM* RNA interference (RNAi) line compared to WT at the same seven timepoints for which phenotypic data were recorded (Figure 5.1 E-F). The *NAM2* homoeologs were expressed at lower levels than *NAM1* (Figure 5.1E-I) with smaller differences between WT and RNAi.

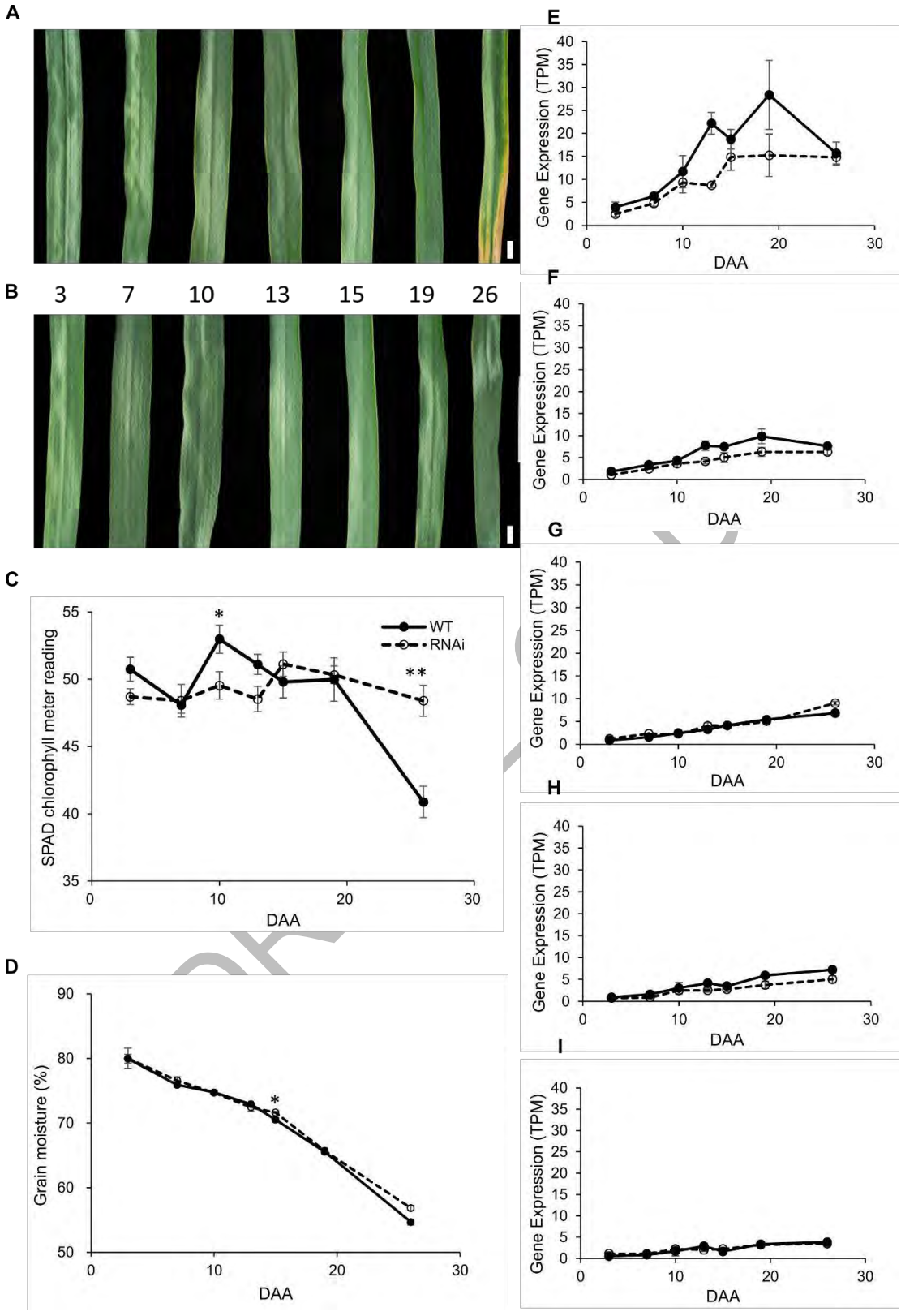

***Figure 5.1. Characterization of wild type (WT) and* NAM-*RNAi plants in time course of flag leaf 252 senescence from 3 to 26 days after anthesis (DAA). A and B) flag leaf images of WT and* NAM *RNAi from 3 to 26 DAA (WT images in A) originally published in Borrill et al., 2019), C) SPAD chlorophyll meter readings for flag leaves across the time course from 3 to 26 DAA, n=5, D) grain moisture content of grains across the time course from 3 to 26 DAA, n=5, E-I; expression pattern of* NAM-1 *and* NAM-2 *genes 3 to 26 DAA in WT and* NAM**

RNAi measured using RNA-Seq. E)* NAM-A1 (TraesCS6A02G108300), *F)* NAM-D1 (TraesCS6D02G096300), *G)* NAM-A2 (TraesCS2A02G201800), *H)* NAM-B2 (TraesCS2B02G228900), *I)* NAM-D2 *(*TraesCS2D02G214100*). Error bars represent standard error of the mean. n=5 for SPAD chlorophyll meter reading and grain moisture content and n=3 for gene expression data. Scale bar = 1 cm.

5.4.2. Transcriptome profile in WT and RNAi during senescence

5.4.2.1. WT plants had stronger transcriptional changes than RNAi during the time course

RNA was extracted from the flag leaf and sequenced for each of the seven time points. RNASeq data were aligned using kallisto (Bray et al., 2016) to the RefSeqv1.1 transcriptome annotation (Appels et al., 2018). Initially we observed artificially high levels of *NAM* gene expression in the *NAM* RNAi samples. Examining the read alignments this was caused by mapping of transcripts from the transgenic *NAM* RNAi construct to the *NAM* genes. Therefore we masked these regions of the coding sequence of the *NAM* genes with Ns to prevented artificial inflation of *NAM* gene expression in the *NAM* RNAi samples (on average 29% of the *NAM* coding sequence was masked). After re-mapping to the RefSeqv1.1 transcriptome with masked regions in the *NAM* genes, on average samples had 33.7M reads and 27.5M reads were pseudoaligned by kallisto (81.3 %) (Supplementary Table 5).

As a first step to understand transcriptional differences between WT and RNAi we compared gene expression at each time point individually. In most timepoints < 80 genes were upregulated in WT compared to RNAi, except at 26 DAA when 549 genes were upregulated (>2-fold change, FDR <0.001; Supplementary Table 6 and 7). The 549 genes upregulated in WT at 26 DAA were enriched for GO terms associated with senescence and chlorophyll catabolism (padj<0.05; Supplementary Table 7). More genes were downregulated than upregulated at every time point, with a range from 99 to 874 downregulated genes. The largest number of downregulated genes occurred at the start and end of the time course. At the earliest timepoint 3 DAA, 693 genes were downregulated in WT compared to RNAi (>2fold change, FDR <0.001; Supplementary Table 7) and these were enriched for GO terms associated with catabolic processes and response to freezing. At the final timepoint 874 genes were downregulated in WT compared to RNAi and these were enriched for GO terms related to photosynthesis. None of the *NAM* genes (Figure 5.1) were identified as differentially expressed between WT and RNAi by DESeq2, which may be due to variability between replicates and stringent p-value and fold change thresholds. Although this pairwise analysis identifies genes differentially expressed at each timepoint, it ignores information from adjacent timepoints and does not provide information on individual gene expression

trajectories over the time course. Therefore we decided to identify DEGs in each genotype separately over time to reveal how dynamic gene expression is affected by the reduction in *NAM* gene expression in the RNAi lines compared to WT. We hypothesised that this approach would identify how the knock-down of *NAM* genes affects the overall senescence transcriptional programme and provide time-specific information.

To identify differently expressed genes in both WT and RNAi, we used ImpulseDE2 and Gradient Tool. We found that from 3 to 26 DAA 6,508 (WT) and 2,605 (RNAi) genes were differentially expressed. In WT out of 6,508 DEGs, 3,870 genes were upregulated and 2,638 genes were downregulated (Figure 5.2; Supplementary Table 1; containing top 500 most significant genes). While in RNAi, out of 2,605 DEGs, 1,585 genes were upregulated and 1,020 genes were downregulated (Figure 5.2; Supplementary Table 2; containing top 500 most significant genes). During the time course, more genes were up-regulated than downregulated in both WT and RNAi. This suggests that senescence is actively controlled through transcriptional upregulation rather than general downregulation in wheat. Approximately half of the DEGs in RNAi were also found in WT (Figure 5.2), contrastingly most DEGs in WT were not differentially expressed in RNAi, suggesting a unique transcriptional response in WT compared to RNAi.

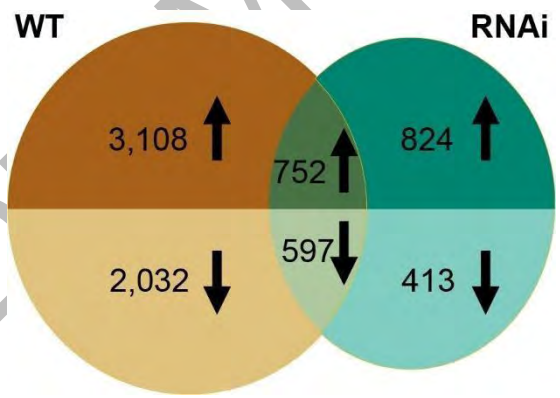

Figure 5.2. Venn diagrams of differentially expressed genes (DEGs) in WT and* NAM *RNAi. Upregulated genes are shown in the top half of each circle and downregulated genes in the bottom half of each circle. The intersection of two circles represents genes differentially expressed in both WT and RNAi. Out of 1,368 common DEGs, 19 genes were upregulated in one genotype and downregulated in the other (not shown).

5.4.2.2. An initial wave of downregulation is followed by upregulation of gene expression in both genotypes

To understand the temporal nature of gene expression changes, we assigned DEGs (6,508 in WT and 2,605 in RNAi) into groups according to the first time they were up-or

downregulated. For instance, a gene first up-regulated at 7 DAA would be grouped as "U07" (up 07 DAA), and a gene first showed down-regulation at this time point would be grouped as "D07". We found that less than 2% of genes were up- and then down-regulated or vice versa during the time course in either WT (1.4%) or RNAi (1.8%) and these were excluded from further analysis. The remaining 98% of genes were described by 13 expression patterns in wild-type and RNAi (Supplementary Table 8).

In WT and RNAi, up-and down-regulation patterns were not evenly spaced over time. In both WT and RNAi, the number of genes upregulated increased during the early time points from 3 to 10 DAA, but from 13 DAA onwards the number of genes upregulated in RNAi fell to a lower level, whereas in WT 13 DAA was the timepoint with the highest number of genes upregulated (Figure 5.3A). Many more genes were first upregulated in WT at later timepoints than in RNAi. With the onset of chlorophyll loss at the end of the time course (26 DAA; Figure 5.1A), very few genes showed differential expression in either WT or *NAM* RNAi (7 genes upregulated in each line). Initiation of downregulation was stronger in the early stages of the time course in both lines, with more genes downregulated in WT than RNAi (Figure 5.3B). As senescence progressed, only a limited number of genes were downregulated; 44 genes at 19 DAA in WT. In both WT and RNAi no gene was downregulated at 26 DAA suggesting that senescence process is actively regulated through transcriptional upregulation at later stages of senescence (Figure 5.3A). A major shift from downregulation at the start of senescence to upregulation enduring the middle and later timepoints is evident in our dataset (Figure 5.3A and 5.3B).

We found that the most of DEGs were up or down-regulated at different timepoints in WT and RNAi. For example, in WT at 3 DAA (U03) 192 genes were upregulated but in RNAi only 62 of these genes were upregulated at 3DAA while 130 of them were not differentially expressed (Not DE; Figure 5.3C). This limited conservation of expression profiles was common across all timepoints and in both up-and down-regulated genes (Figure 5.3C). We identified 1,209 genes which were not differentially expressed in WT but showed differential expression in RNAi and an even greater number were differentially expressed in WT but not differentially expressed in RNAi (5,055 genes; Figure 5.3C).

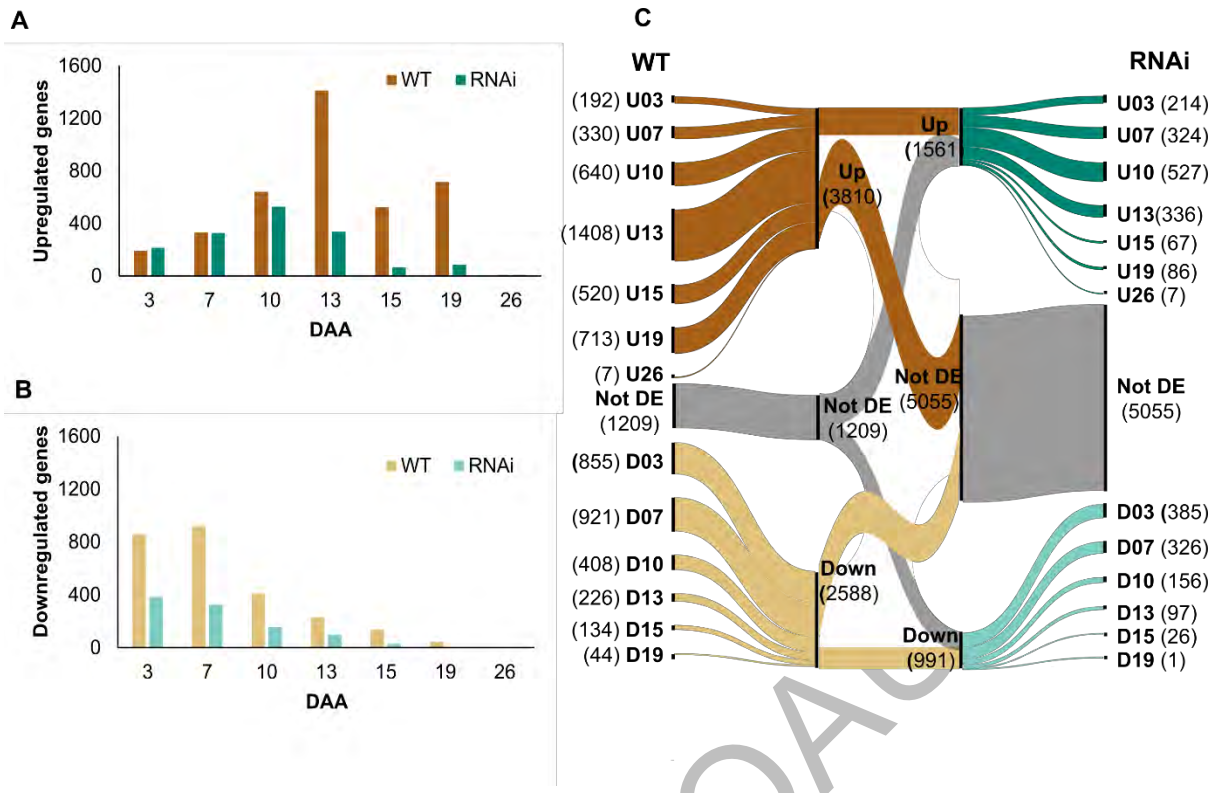

Figure 5.3. Differential expression of genes across the time course in WT and* NAM *RNAi plants. Genes are grouped according to the first time they were up-or downregulated. A) upregulated genes, B) downregulated genes, C) alluvial plot showing comparison of differential expression patterns in WT and RNAi. In C) the number in brackets for each group pattern represents number of DEGs at that time point. Not DE stands for not differentially expressed.

5.4.3. Gene Ontology (GO) term enrichments in WT and RNAi

To identify the biological processes and functions associated with each group pattern in our dataset we performed GO enrichment analysis (Figure 5.4; Supplementary Table 8). DEGs in WT were more strongly enriched for GO terms associated with hormones, nitrogen metabolism and other nutrient metabolism than DEGs in RNAi (Figure 5.4). Upregulated genes were enriched for hormone signalling and biosynthesis genes in WT but not in RNAi (Figure 5.4A). Up-regulated genes were enriched for GO terms associated with protein transport, proteasome, vesicle mediated transport and expressed at later time points in WT compared to RNAi (Figure 5.4D). Genes enriched for GO terms associated with housekeeping functions such as chloroplasts, photosynthesis, rRNA processing, and translation were downregulated at more timepoints in WT compared to RNAi (Figure 5.4D).

The differential expression patterns of genes enriched with N-associated GO terms were more obvious in WT than RNAi. GO terms related to Nitrogen (N) metabolism such as

nitrogen and amino acid transport, glutamine, glutamate, cysteine biosynthesis were mostly downregulated early in the time course and then upregulated in both WT and RNAi, although the upregulation was less extensive in RNAi (Figure 5.4B). Genes enriched with GO terms associated with other nutrients such as copper, phosphate, potassium, and zinc showed upregulation in WT but most of them were not enriched in RNAi except zinc at 13 DAA (Figure 5.4C). Genes enriched with GO terms associated with metal ion transport were downregulated at early time points in both WT and RNAi (Figure 5.4C). Overall, DEGs in WT had stronger GO term enrichments, with particularly strong enrichment for processes related to hormones and nitrogen metabolism, but these enrichments were less frequently observed in RNAi.

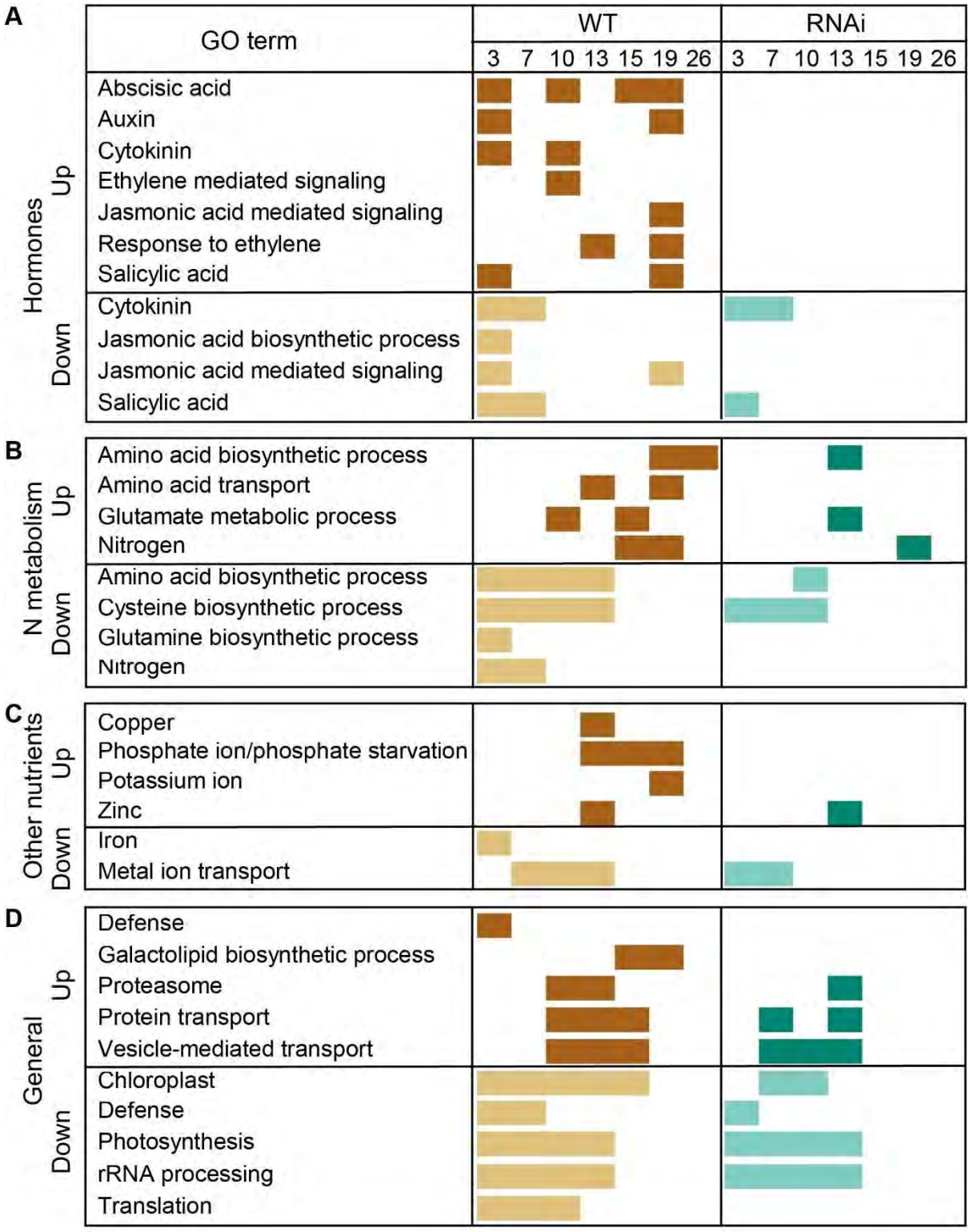

Figure 5.4. Biological processes enriched in up and down-regulated genes in wild type (WT) and RNAi lines during a time course (3-26 DAA) of senescence. Filled rectangles indicate that genes starting to be differentially expressed at that time point are enriched for that specific gene ontology (GO) term. Enriched GO terms are grouped into A) Hormones, B) Nitrogen (N) metabolism, C) Other nutrients and D) General processes. Brown rectangles represents up-regulated genes in WT; dark green represents up-regulated genes in RNAi;

pale yellow rectangles represent downregulated genes in WT and light green rectangles represent down-regulated genes in RNAi.

5.4.4. Genes directly involved in nitrogen metabolism

In order to identify effect of *NAM* gene on nitrogen metabolic pathway during time course of senescence, we assembled the list of genes involved in nitrogen metabolism in Arabidopsis through previous literature searches. We then identified their respective orthologs in wheat (*Triticum aestivum L.*) using *EnsemblPlants* ortholog information downloaded via BioMart. After that, we identified the expression patterns of genes involved in nitrogen transport, assimilation remobilization and transcriptional regulation in WT and RNAi lines. In total we identified 1,027 genes in wheat associated with nitrogen metabolism, of which 587 and 580 genes were expressed during flag leaf senescence in WT and RNAi, respectively. Nitrogen associated genes were differentially expressed more in WT (136) than RNAi (41) during the time course. The greater number of nitrogen associated genes DEGs in WT suggests greater changes to nitrogen remobilization or metabolism in WT than RNAi. Overall, nitrogen associated genes expressed during time course of senescence showed upregulation in WT but most of them were downregulated or not differentially expressed in *NAM* RNAi line indicating that reduced *NAM* genes affects the expression patterns of these genes in wheat.

5.4.4.1. Expression patterns of nitrogen transporters in WT and RNAi

During senescence, nitrogen is transported via *ammonium* (*AMT2;1*) and *nitrate* (*NRT1.4, NFP5.10, NRT2.5*) *transporters* across the cell membrane in the form of nitrate ($NO3^-$) and ammonium ($NH4^+$) ions (Kong et al., 2016; van der Graaff et al., 2006). Most nitrate transporters in our dataset were upregulated in WT flag leaves but not differentially expressed in *NAM* RNAi (Supplementary Table 3 and 9). Similarly, the highly expressed ammonium *transporter* (*AMT2; 1*; *TraesCS4A02G352900*) was upregulated in WT but not differentially expressed in RNAi during our time course (Figure 5.5A).

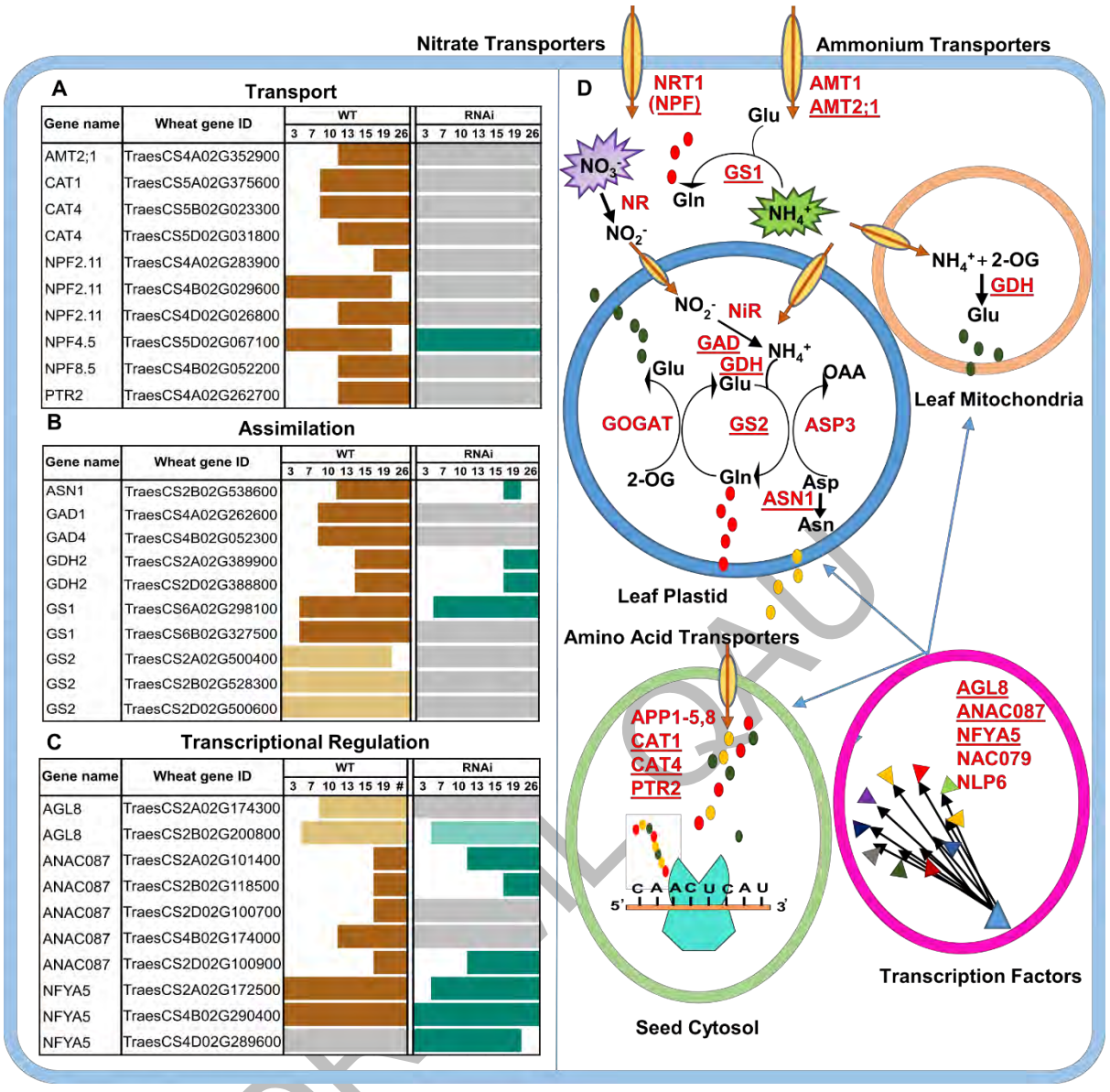

Figure 5.5. Schematic representation of genes, enzymes and processes involved in nitrogen metabolisms in wheat. The plots present on left side of figure represent differential expression pattern of the ten most highly expressed genes involved in nitrogen cycling for each category: A) transport, B) assimilation and C) transcriptional regulation. These genes are red coloured, bold and underlined in the figure to the right (D). Gene names (A-C) are given based on orthology to Arabidopsis and orthology is not always 1:1 between Arabidopsis and wheat (Supplementary Table 3). In A-C) brown rectangles represents up-regulated genes in WT; dark green represents up-regulated genes in RNAi; pale yellow rectangles represent down-regulated genes in WT and light green rectangles represent down-regulated genes in RNAi. D) Nitrogen associated gene pathways in wheat. Ammonium (AMTs) and nitrate transporters (NRTs) transport ammonium ($NH4^+$) and nitrate ions ($NO3^-$) across the cell membrane. In the cytosol, Nitrate reductase (NR) enzyme reduces nitrate to nitrite. Then nitrite reductase (NiR) reduces nitrite into ammonium in the plastids. After that Glutamine synthetase (GS)/glutamine-2-oxoglutarate aminotransferase (GOGAT) cycle assimilates ammonia into N-containing compounds. Asparagine synthetase (ASN), and glutamate dehydrogenase (GDH) are involved in further assimilation of nitrogen compounds into different amino acids. Glu, glutamate; Gln, glutamine; Asn, asparagine; Asp, aspartate; 2-

OG, 2-oxoglutarate; OAA, oxaloacetate. These amino acids are then transported to developing grain through different amino acid transporters (AAP, CAT1, CAT4, PTR2). All these steps are regulated by transcription factors (AGL8, ANAC087, NFYA5, NAC079, NLP6).

The deaminating activity occurring in the senescing leaf provides glutamine (*Gln*), glutamate (*Glu*) and asparagine (*Asn*) which are then transported to the seed via amino acid transporters. These amino acid transporters include permeases (*AAPs*), proline transporters (*ProTs*), *ANT1*like aromatic, and neutral amino acid transporters, γ-aminobutyric acid transporters (GATs) cationic amino acid transporters (*CATs*) and lysine-histidine-like transporters (*LHTs*). The amino acid transporters *CAT1* (*TraesCS5A02G375600), CAT4 (TraesCS5B02G023300, TraesCS5D02G031800), NPF2.11(TraesCS4A02G283900, TraesCS4B02G029600, TraesCS4D02G026800), NPF8.5 (TraesCS4B02G052200)* and *PTR2 (TraesCS4A02G262700)* were upregulated in WT but were not differentially expressed in RNAi (Figure 5.5A). Interestingly, *NPF4.5(TraesCS5D02G067100)* was the only amino acid transporter among ten highly expressed nitrogen transporters which was upregulated in both WT and RNAi (Figure 5.5A). Many other important nitrogen transporters were also expressed in our data either in WT or RNAi such as *AAP (AAP2, AAP3, AAP4* and *AAP8), PTR, CAT, GAT, LAT, LHT, ANT1* and *NPF*. Most of these amino acid transporters showed upregulation in WT but these were either not differentially expressed or down-regulated in RNAi (Figure 5.5A; Supplementary Table 3 and 9).

5.4.4.2. Expression patterns of nitrogen assimilation genes in WT and RNAi

Many genes known to be involved in nitrogen assimilation and remobilization were expressed in our RNA seq data (Figure 5.5B, Supplementary Table 3 and 10) such as *nitrate reductase* (*NIA*), *nitrite reductase* (*NR*), *glutamine synthetase* (*GS*), *glutamate dehydrogenase* (*GDH*), *glutamate decarboxylase* (*GAD*) and *asparagine synthetase* (*ASN*). In general nitrogen assimilation and remobilisation related genes were more frequently up or downregulated in the WT time course than in the RNAi time course (Figure 5.5B). Some genes showed later upregulation in RNAi than in WT including *ASN1 (TraesCS2B02G538600) and GDH2 (TraesCS2A02G389900, TraesCS2D02G388800)*. Other genes were up-regulated in WT but not differentially expressed in RNAi including *GAD1* (*TraesCS4A02G262600*) and *GAD4 (TraesCS4B02G052300).* Both *GS1* homoeologs *(TraesCS6A02G298100 and TraesCS6B02G327500)* were upregulated in WT, but only the A homoeolog was upregulated in RNAi. Three homeologs of *GS2* (*TraesCS2A02G500400, TraesCS2B02G528300* and

TraesCS2D02G500600) were down-regulated in WT but not differentially expressed in RNAi (Figure 5.5B).

5.4.4.3. Expression patterns of nitrogen transcriptional regulators in WT and RNAi

In addition to the transporters and enzymes, a number of regulatory TF genes are known in Arabidopsis to participate in nitrogen metabolism. In our dataset, the A homoeolog of *AGL8 (TraesCS2A02G174300)* was down-regulated in WT but not differentially expressed in RNAi, while its B homeolog *(TraesCS2B02G200800)* showed down-regulation in both WT and RNAi (Figure 5.5C; Supplementary Table 3 and 11). For *ANAC087*, the five orthologs were upregulated in WT while two of them (*TraesCS4B02G174000* and *TraesCS2D02G100700*) were not differentially expressed in RNAi (Figure 5.5C). Overall, we found that many more nitrogen associated genes were up or downregulated during the senescence time course in wild type than in *NAM* RNAi plants (Figure 5.5A-D and Supplementary Table 12).

5.5. Discussion

In this study, we compared transcriptional changes in wild type and *NAM* RNAi wheat plants associated with flag leaf senescence. We found that approximately 2.5 times more genes were differentially expressed in wild type than in RNAi plants from 3 to 26 days after anthesis. Many genes associated with nitrogen metabolism are differentially expressed in wild type plants but not in RNAi plants, which is consistent with previously reported phenotypic effects of *NAM* genes on nitrogen remobilisation (Uauy et al., 2006; Waters et al., 2009).

5.5.1. Dynamic transcriptional changes uncovered through time-aware differential expression analysis

The conventional approach to understand the transcriptional responses to a gene requires pairwise comparison between plants with and without the gene of interest. Using DESeq2 we carried out this pairwise analysis and identified tens to hundreds of genes differentially expressed between wild type and RNAi plants at each timepoint during senescence. Our findings were consistent with previous analyses of *NAM* RNAi and *NAM* mutant lines, including identifying changes to photosynthetic genes (Cantu et al., 2011; Pearce et al., 2014). However, specialised analysis techniques for time courses allow information to be shared between timepoints, which allows a more accurate and powerful analysis for datasets

with larger numbers of timepoints. To take advantage of this we analysed transcriptional changes across our seven timepoints from 3 to 26 DAA in each genotype.

We found that although 52,395 (WT) and 52,626 (RNAi) genes were expressed in senescing flag leaves, only 6,508 (WT) and 2,605 (RNAi) genes were differentially expressed during this time period. In both genotypes, more genes were upregulated than downregulated, which shows that senescence is an actively regulated developmental process, as has been previously reported for wheat and other plant species (Borrill et al., 2019; Breeze et al., 2011; Zhang et al., 2018). Most of the genes differentially expressed in wild type plants were not differentially expressed in *NAM* RNAi plants (5,140/6,508), suggesting that *NAM* genes control approximately three-quarters of the transcription response during these early stages of senescence. We observed that WT and RNAi DEGs were split into two waves of transcriptional changes with an initial wave of downregulation followed by upregulation during later timepoints, which might not have been evident from a less time-resolved data set. *NAM* RNAi plants maintain these transcriptional waves during senescence, albeit to a lesser extent than wild type, which indicates that some transcriptional changes during senescence are *NAM*-independent, as previously proposed by Pearce et al. (2014). Nevertheless, the *NAM*-independent DEGs are much lower in number than DEGs in the wild type time course, confirming that *NAM* genes play a major role in the transcriptional regulation of early senescence in wheat (Cantu et al., 2011; Harrington et al., 2020; Pearce et al., 2014).

DEGs in WT were more strongly enriched for GO terms associated with hormones, nitrogen metabolism and other nutrient metabolism than DEGs in RNAi (Figure 5.4). Overall genes enriched with GO terms relating to nitrogen metabolism and nutrition showed up- and downregulation in WT but most of these genes were not differentially expressed in *NAM* RNAi. This is consistent with analysis at 12 days after anthesis which identified that genes annotated to be involved in protein metabolism and catalytic process were mostly upregulated at 12 DAA in wild type compared to *NAM* RNAi wheat (Cantu et al., 2011).

5.5.2. Effect of *NAM* genes on nitrogen remobilization

Previous studies have shown that *NAM* genes affect grain protein content by altering nitrogen remobilisation in a range of genetic backgrounds and environmental conditions (Alhabbar et al., 2018; Avni et al., 2014; Pearce et al., 2014; Uauy et al., 2006; Waters et al., 2009), yet how this is mediated at the gene expression level is less well understood. To address

this, we identified nitrogen metabolism associated genes in the RefSeqv1.1 gene annotation. In total we identified 1,027 genes which may be involved in nitrogen transport, assimilation remobilization or transcriptional regulation in wheat by orthology to Arabidopsis. Approximately half of these genes were expressed in our flag leaf time course in each genotype. Over three times more nitrogen associated genes were differentially expressed in WT than in RNAi across the time course (136 vs 41 genes, respectively) indicating that reduced expression of *NAM* genes affects nitrogen remobilisation at the transcriptional level during senescence. The differences in nitrogen associated gene expression between WT and RNAi may be due to direct or downstream effects of *NAM* genes which could be tested in the future using ChIP-seq or DAP-seq approaches.

We found that *NAM* genes play a significant role in controlling the expression pattern of genes associated with nitrogen transport during senescence in wheat. For example orthologs of *AAP8* (*TraesCS7B02G271151* and *TraesCS7D02G366000*) were upregulated from 10 and 13 DAA in WT, but not in RNAi. These genes had been previously shown to be highly expressed during later stages of grain development (28-30 days post anthesis; *TaAAP21*), but their potential role in the flag leaf was not noted because flag leaf samples examined were from earlier developmental stages (Wan et al., 2017; Wan et al., 2021). Manipulating these amino acid transporters has the potential to improve grain yields, nitrogen use efficiency, and protein content in crops (Dellero, 2020), and those which are *NAM* regulated (i.e. upregulated in WT but not RNAi) represent a good starting point for precise functional studies. Overall, many nitrogen transport genes were upregulated in WT but were not differentially expressed in the RNAi lines, which may indicate a true absence of transcriptional responsiveness in the RNAi line or alternatively these responses may be delayed in the RNAi line. Our analysis indicates that the widespread changes to gene expression in RNAi compared to WT are not merely a delay in timing of changes, but instead represent a loss of many transcriptional responses (Supplementary Table 3).

Other nitrogen associated genes showed similar trends to the transporters, with more genes differentially expressed in WT than in RNAi. For example the B homoeolog of core nitrogen assimilation gene *glutamine synthetase 1* (*GS1*) (*TraesCS6B02G327500*) was upregulated in WT but not RNAi, however the A homeolog (*TraesCS6A02G298100*) was upregulated in both WT and RNAi but to a higher maximum level in WT than RNAi. The upregulation of the A homoeolog in the RNAi as well as the WT, is consistent with *NAM* RNAi lines still being able to remobilise some nitrogen, albeit to a lower degree than WT

(Waters et al., 2009) and with previous reports of the A homoeolog being more highly expressed than other homoeologs (Wei et al., 2021). We found that *glutamine synthetase 2* (*GS2*) was downregulated during senescence in WT, consistent with a previous study under high and low nitrogen (Wei et al., 2021). However, *GS2* was not differentially expressed in RNAi, which might indicate a loss of transcriptional control in the RNAi line across the nitrogen assimilation pathway, or a compensatory mechanism to increase nitrogen cycling.

We identified putative wheat orthologs of Arabidopsis transcription factors which are associated with nitrogen remobilisation. However, for this set of genes the differences between WT and RNAi at the gene expression level were weaker than for nitrogen transporters or assimilation genes, suggesting either that transcription factors controlling the nitrogen pathway are less affected by *NAM* genes, or that transcription factors regulating this process are not conserved between Arabidopsis and wheat. We previously found that NAC transcription factors which control senescence in Arabidopsis are not well conserved at the expression level in wheat during senescence (Borrill et al., 2019), therefore it seems likely that regulatory genes are also poorly conserved in nitrogen remobilisation. Combining the differentially expressed transcription factors identified in this study with transcription factors which respond to different levels of nitrogen application (Effah et al., 2022) may provide a fruitful avenue to prioritise candidate genes for functional characterisation.

5.6. Conclusions

The use of time-aware differential expression analysis allows detailed analysis of the dynamics of gene expression during a developmental process such as monocarpic senescence. Here, we found that wild type plants undergo stronger transcriptional changes immediately after anthesis, than *NAM* RNAi lines with delayed senescence, including genes associated with nitrogen metabolism. Nevertheless, *NAM* RNAi lines do show some gene expression changes which are associated with senescence, indicating that there are *NAM*-independent pathways which regulate senescence in wheat. The list of putative *NAM*-regulated genes generated in this study provides a valuable entry point to dissect the pathways regulating senescence and nutrient translocation in wheat.

Chapter #6

Conclusion

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Advances in plant-omics in the last two decades have demonstrated an unprecedented power to dissect the genetic basis of important agronomic traits. The advent of next generation sequencing platforms and their utilization in breeding have helped breeders to jump from QTL mapping to association mapping, from marker trait selection to genomic selection, from years to days, small region sequencing to complete genome sequencing and most importantly from millions of dollars to hundreds of dollars. Transcriptomics of plants using next generation platforms have also helped to understand not only complete transcriptional responses of plants but also post transcriptional responses. With escalating temperature, intense and frequent heat waves, water scarcity, and nutrient deficient soils together with soaring population rates, breeders needs to utilize plant-omics tools to tailor cultivars to ensure future food security and safety. In developing countries, crop productivity is mainly limited by poor access to nitrogen fertilizer. However, substantial increase in the use of N fertilizer positively increases crop productivity in affluent countries over recent decades.

The overuse of N fertilisers in recent decades has resulted in unfavourable soil and environmental degradations such as acidification, N leaching into groundwater, and greenhouse gas (N_2O) emissions. Crop yields have declined in areas with high soil acidification due to a lack of major nutrients and basic cations, as well as the toxic effect of acidic cations. Excessive N fertilisation also raises fertiliser costs, reduces N-use efficiency, and has a negative impact on livestock and humans. Moreover, fertilizer prices have been continuously rising since 2020, reaching an all-time high in the fall of 2021 in global market. To lessen excessive N fertilization, efficient use of nitrogen is need of time. The demand for nitrogen at global level is currently up to 117 million metric tons, with an expected 1.5% increase annually in coming years (FAO, 2019). Therefore, the management of nitrogen use efficiency is necessary to achieve high crop yield at the current time.

- ✓ The purpose of this study is to examine the roles of yield contributing traits in low and high N input environments. This work proved reliability and the power of multi-locus (ML)-GWAS models such as FarmCPU about N related traits in wheat and provided new insights into the understanding of the N pathway in wheat, which may facilitate breeding in wheat by using non-destructive precision agriculture approaches for efficient utilization of N in bread wheat. To

identify genomic regions associated with yield determining traits in historical bread wheat panel of Pakistan and then comparing with the wheat reference genome helped to identify potential candidate genes involved in nitrogen pathway in wheat. Identified putative candidate genes associated with significant MTAs, may be directly or indirectly involved with various biological processes, molecular functions and cellular component organization associated with nitrogen pathway.

- ✓ In this study, correlation (r) and path (β) coefficients among grain yield components and root traits with grain yield were computed to use them as selection criteria for grain yield. However, based on the results of path-coefficient analysis, it could be concluded that tiller per plant (TpP), days to maturity (DM), nitrogen use efficiency (NUE) and root length (RL) were the most important traits. Hence, these traits could be use as indirect selection criteria to improve grain yield under varying N-levels. This approach provides wheat breeders with an opportunity to produce high yielding cultivars with preferred combinations of yield components.
- ✓ The current findings can be used to investigate the role of nitrogen fertilizer in lowering crop canopy temperature at the molecular level. In the last decade, many omics approaches have transformed research strategies that plant biotechnologists and breeders have used to investigate underlying abiotic stress tolerance mechanisms. There is an urgent need for a deeper understanding of nutrient-use and heat-stress-tolerance mechanisms of different wheat varieties at the transcriptomic level. The use of genomics, proteomics, metabolomics, and transcriptomics data sets are needed rather than relying on phenomics data sets only.
- ✓ The use of time-aware differential expression analysis allows detailed analysis of the dynamics of gene expression during a developmental process such as monocarpic senescence. Here, we found that wild type plants undergo stronger transcriptional changes immediately after anthesis, than *NAM* RNAi lines with delayed senescence, including genes associated with nitrogen metabolism. Nevertheless, *NAM* RNAi lines do show some gene expression changes which are associated with senescence, indicating that there are *NAM*-independent pathways which regulate senescence in wheat. The list of putative *NAM*-regulated genes generated in this study provides a valuable entry point to dissect the pathways regulating senescence and nutrient translocation in wheat.

The current thesis reports fundamental knowledge of molecular basis of nitrogen response in bread wheat. Moreover, we demonstrated that utilization of multiple plant-omics approaches will allow the identification of robust candidates for agronomical quantitative traits related to nitrogen in bread wheat. The identified genes/loci can be functionally validated using

transgenic as well as non-transgenic approaches and can be consider as molecular markers for genomics/marker selection breeding programs.

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APPENDIX

Appendix

Appendix 2.1. Pedigree of the panel used for phenotyping and statistical investigation

SR. NO.	VARIETY NAME	PEDIGREE
1	KOHSAR-95	PSN/BOW
2	SHAHKAR-95	CNO67//SN64/KLRE/3/8156
3	BAKHTAWAR-94	BB/NOR67
4	KAGHAN-93	CMH-77A917/PKV 1600//RL6010/6*SKA
5	SAIRAB-92	CHENAB2000/INQ-91
6	ANMOL-91	LUAN/KOH-97
7	PARWAZ-94	OASIS/SKAUZ//4*BCN/3/2*PASTOR
8	PASBAN-90	BLS/KHUSHAL
9	MARGHALA-99	OPATA/BOW'S'
10	DERA	F12-71/COC/CNO 79
11	DAMAN	BOWS/3/CAR853/COC//VEES
12	D-97	FORD//DUNDEE/BOBIN or FORD/DONDEE (I)
13	KOHISTAN-97	KVZ/3/TOB/CTFN/BB/4/BLO/5/VEE#5/6/BOW/3/YD//BB/CHA
14	MH-97	KAUZ/PASTOR
15	NARC-2009	INQALAB 91*2/TUKURU
16	SULEMAN -96	BUC/FLK//MYNA/VUL
17	NOWSHERA-96	C516/C591
18	ROHTAS-9O	INIA F 66/TH.DISTICHUM//INIA F 66/3/GENARO T 81 or INIA F 66/ A.DISTCHUM//INIA66/3/GEN
19	SOGHAT-90	PSN/BOW
20	BWP-97	NORD-DESPREZ(ND)/VG-9144//KALYANSONA/BLUEBIRD/3/YACO/4/VEERY-5
21	DWR-97; DRAWAR 97	SASONO KOMOGI/NORIN//BOB'S'
22	SHALIMAR-88	WL 711/CROW"S"
23	KHYBER-87	21931-CHAPINGO53/ANDES SIB/3/Y50/4/C271
24	RAWAL-87	MAYA/MON//KVZ/TRM
25	SUTLAJ-86	ULC/PVN//TAN/3/BUC
26	PUNJAB-85	BURGUS/SORT 12-13//KAL/BB/3/PAK 81
27	FSD-85	CHIL/2*STAR
28	FSD-83	MAYA/MON//KVZ/TRM
29	KOHINOOR-83	PT'S'/3/TOB/LFN//BB/4/BB/HD-832-5//ON/5/G-V/ALD'S'//HPO
30	SARHAD-82	JUP/ALD'S'//KLT'S'/3/VEE'S'/6/BEZ//TOB/8156/4/ON/3/6*TH/KF//6*LEE/KF/5
31	PUNJAB-81	PBW65/2*Pastor
32	PAK-81	FURY//KAL/BB
33	ZARDANA	PJ/GB55 or PJ62/GB55
34	ZARGHOON-79	CC/INIA/3/TOB/CTFN//BB/4/7C
35	BWP-79	CNO/LR64A*2//SN64/SN63 or CNO/LR64*2/SON64/SON
36	DIRIK	PIT/GB//C271
37	TARNAB-73	T9/8D or T9 X 8A
38	LYP-73	BB/NOR67

39	PARI-73	FORLANI/ACC//ANA or Fln/ACS//ANA
40	SA-72	C-271/WILLET-DWARF//SONORA-64
41	B-SILVER	C 230 X IP 165
42	CHENAB-70	HARD FEDERATION X 9D
43	YECORA-70	BUC'S'/FCT'S'
44	NURI-70	HARD FED/9D
45	UP-262	land races
46	LOCAL-WHITE	BB/GLL/3/GTO/7C//BB/CNO67
47	POTHOWAR	ATTILA/3/HUI/CARC//CHEN/CHTO/4/ATTILA
48	SA-75	CHUM18/BAU
49	SA-42	C 209 X C 591
50	KHUSHALL-69	II53-388/AN//YT54/N10B/3/LR64/4/B4946.A.4.18.2.IY/Y53//3*Y50
51	WL-711	S308/CHRIS//KAL
52	MEXIPAK	PJ/GB55 or PJ62/GB55
53	SONALIKA	SASONO KOMOGI/NORIN//BOB'S'
54	SANDAL	T9 X 8A
55	LU-26	BLS/KHUSHAL
56	PUNJAB-76	NAI60/CB151//S949/3/MEXIPAK
57	BARANI-70	CNO/LR64A*2//SN64/SN63 or CNO/LR64*2/SON64/SON
58	CHAKWAL-86	KVZ/TRM//PTM/ANA
59	PIRSBK-91	KAUZ//ALTAR84/AOS
60	INQILAB-91	V-1562//CHRC'S'/HORK/3/KUFRA-I/4/CARP'S'/BJY'S'
61	CHAKWAL-97	INIA F66/TH.DISTICHUM//INIAF66/3/GENARO T81 or INIA F66/A.DISTCHUM//INIA66/3/GEN
62	BARANI-83	DWL5023/SNB//SNB
63	CHAKWAL-50	F6.74/BUN//SIS/3/VEE#7 or F6-74/BUN//SIS/3/VEE#7
64	C-217	KHP/D31708//CM74A370/3/CNO79/4/RL6043/4*NAC or KHP/D31708//CM74A370/3/CIANO79/4/RL6043/*4NAC
65	C-228	KVZ/TRM//PTM/ANA
66	C-271	C-230/IP-165;
67	C-273	C-591/C-209; C-209/C-591
68	C-250	CROW'S'/NAC//BOW'S'
69	C-306	AU/UP301//GLL/Sx/3/PEW S/4/MAI S/MAY A S//PEWS
70	C-518	SH-88/90A-204//MH97
71	T-8	land races
72	SKD-1	LU 26/HD 21790/ 2*INQALAB 91
73	TD-1	BY/MAYA/4/BB//HD832.5.5/ON/3/CNO67/PJ62 or PITIC-62/FROND//MEXIPAK/3/PITIC-62/MAZOE-79-75-76 [wheatpedigree.net] or PI/FRND//MXP/3/PI/M20/79
74	RASKOH	Kauz/Yaco//Kauz
75	SARSABZ	TTR/JUN
76	SASSUI	HD-2329
77	WAFAQ	Kauz/Yaco//Kauz
78	AS-2002	CHAM6//KITE/PGO
79	T-9	land races
80	GA-2002	NAI60/CB151//S949/3/MEXIPAK
81	UFAQ	NAI60/CB151/S949/3/MEXIPAK
82	BAKHAR-2002	URES/BOW`S

83	MOOMAL-2003	CNO67//SN64/KLRE/3/8156
84	SH-2003	AU//KAL/BB/3/WOP
85	PIRSBK-04	KAUZ/STAR
86	IMDAD-05	CHILL/2* STAR/4/BOW//BUC/PVN/3/2*VEE#10
87	PIRSBK-05	MUNIA/CHTO//AMSEL
88	SEHER-2006	WL711//F371/TRM
89	SHAFAQ-2006	PB81/HD2182//PB81
90	LASANI-2008	PAVON MUTANT-3
91	PIRSBK-08	JUP/ALD'S'//KLT'S'
92	FSD-08	PBW65/2*Pastor
93	MAIRAJ-08	WT(E)/SON64
94	NARC-09	INQALAB 91*2/TUKURU
95	NARC-11	CNO67/8156//TOB66/CNO67/4/NO/3/12300//LR64A/8156/5/PVN or CNO67/8156//TOB 66/CNO67/4/NOROESTE F66/3/12300//LR64A/8156/5/PVN
96	AARI-11	OPATA/RAYON//KAUZ
97	AAS-11	LU26/HD 2179
98	PUNB-11	CNO67//SON64/KLRE/3/8156
99	FAREED-2006	INQALAB-91/FINK'S'
100	IQBAL-2000	BURT/KENYA//QUETA(L)/3/NAD63
101	FK.SARHAD	MUNIA/CHTO//AMSEL
102	KHIRMAN-2006	ULC/PVN//TAN/3/BUC
103	MANTHAR-2003	KAUZ//ALTAR84/AOS
104	SALEEM-2002	C271//LR64/SN64
105	TATARA-96	CNO//SN64/KLRE/3/8156
106	CHENAB-2000	AMSEL/ATTILA//INQ-91/PEW'S'
107	BWP-2000	NAI60/CB151/S949/3/MEXIPAK
108	AUQAB-2000	INIA/3/SN64/P4160(E)//SN64 or INIA/3/SON64/P4160(E)//SON64
109	BARS-2009	MAI'S'/NORTENO65/H68
110	SH-2002	INQALAB-91/FINK'S'
111	SALEEM-2000	KVZ/TRM//PTM/ANA
112	MILLAT-2011	NOR67/7C
113	MARVI-2000	PB85/NKT'S'
114	SOKOLL	Synthetic Derivative Variety
115	AUR-809	Advance line
116	TAX 8A	Advance line
117	UAF-9452	Advance line
118	V-070 96	Advance line
119	PAVON	VCM//CNO/7C/3/KAL/BB
120	HAIDER-2000	CHIL/WUH3
121	ZARLASHTA-99	URES/BOW'S'
122	PAKISTAN-13	CMH84.3379/CMH78.578//MILAN
123	SHAKAR-13	CMH84.3379/CMH78.578//MILAN
124	C-591	PRL/PASTOR//2236(V6550/SUTLEH-86)

Appendix 2.2. Genome-wide association mapping showing marker trait association at –log10 (p) > 3

Trait	Method	Marker	Chrom	Position	p Value	-log10(p)
CHL_C	FarmCPU	GENE-0014_142	1A	1211191	0.00055733	3.26
CHL_C	FarmCPU	RAC875_c9457_788	1A	6655226	0.00019169	3.72
CHL_C	FarmCPU	RAC875_c37934_225	1A	20977313	0.00055733	3.26
CHL_C	FarmCPU	GENE-0065_246	1A	57191255	0.00055733	3.26
CHL_C	FarmCPU	BS00062723_51	1A	58704218	0.00055733	3.26
CHL_C	FarmCPU	Tdurum_contig14418_753	1A	62615559	0.00055733	3.26
CHL_C	FarmCPU	Tdurum_contig14418_1242	1A	62616559	0.00037408	3.43
CHL_C	FarmCPU	BobWhite_c15522_250	1A	64693355	0.00055733	3.26
CHL_C	FarmCPU	RAC875_c19348_713	1A	74437124	0.00055733	3.26
CHL_C	FarmCPU	BS00110181_51	1A	82531647	0.00055733	3.26
CHL_C	FarmCPU	Excalibur_c14541_350	1A	91058643	0.00055733	3.26
CHL_C	FarmCPU	GENE-0564_313	1A	91594191	0.00055733	3.26
CHL_C	FarmCPU	CAP8_c2036_140	1A	94245212	0.00055733	3.26
CHL_C	FarmCPU	GENE-0489_946	1A	95857446	0.00055733	3.26
CHL_C	FarmCPU	Tdurum_contig42247_2504	1A	96473400	0.00055733	3.26
CHL_C	FarmCPU	BS00063964_51	1A	127076766	0.00055733	3.26
CHL_C	FarmCPU	Tdurum_contig13709_317	1A	132414787	0.00055733	3.26
CHL_C	FarmCPU	IAAV1625	1A	133416845	0.00055733	3.26
CHL_C	FarmCPU	GENE-0553_173	1A	147354967	0.00055733	3.26
CHL_C	FarmCPU	Kukri_c33586_75	1A	149874043	0.00055733	3.26
CHL_C	GLM	RAC875_c9457_788	1A	6655226	0.00024965	3.61
CHL_C	GLM	Tdurum_contig14418_1242	1A	62616559	0.00061943	3.21
CHL_C	MLM	RAC875_c9457_788	1A	6655226	0.00069167	3.17
CHL_T2	FarmCPU	BS00081002_51	1A	535434824	3.62E-05	4.45
CHL_T2	GLM	BS00081002_51	1A	535434824	8.40E-05	4.08
CHL_T2	MLM	BS00081002_51	1A	535434824	0.00017897	3.75
FLA_T1	FarmCPU	IAAV5931	1A	54022578	0.00090109	3.05
FLA_T1	FarmCPU	wsnp_BE445121A_Ta_1_8	1A	54353607	0.00090109	3.05
FLA_T1	FarmCPU	RAC875_c37183_331	1A	66893592	0.0007535	3.13
FLA_T1	FarmCPU	BS00030644_51	1A	138228904	0.0005025	3.3
FLA_T1	FarmCPU	BS00076538_51	1A	180412596	0.00090109	3.05
FLA_T1	FarmCPU	Ku_c2898_1284	1A	182069500	0.00041505	3.39
FLA_T1	FarmCPU	wsnp_Ex_c13724_21535046	1A	185141694	0.00090109	3.05
FLA_T1	FarmCPU	Kukri_rep_c81206_235	1A	208681924	0.00025803	3.59
FLA_T1	FarmCPU	IAAV742	1A	246129945	0.00066461	3.18
FLA_T1	FarmCPU	wsnp_Ex_c18196_27006489	1A	249053411	0.0008454	3.08
FLA_T1	FarmCPU	BS00021895_51	1A	270007846	0.00081078	3.1
FLA_T1	FarmCPU	BS00021730_51	1A	272313191	0.00081078	3.1
FLA_T1	FarmCPU	BS00048887_51	1A	274785571	0.00081078	3.1
FLA_T1	FarmCPU	GENE-0287_28	1A	276971953	0.00081078	3.1
FLA_T1	FarmCPU	BS00065930_51	1A	289328523	0.00021114	3.68
FLA_T1	FarmCPU	CAP8_c806_297	1A	297380892	0.00081078	3.1

FLA_T1	FarmCPU	BS00064679_51	1A	311806302	0.00060082	3.23
FLA_T1	GLM	IAAV5931	1A	54022578	0.00012644	3.9
FLA_T1	GLM	wsnp_BE445121A_Ta_1_8	1A	54353607	0.00012644	3.9
FLA_T1	GLM	RAC875_c37183_331	1A	66893592	0.00062514	3.21
FLA_T1	GLM	wsnp_Ex_c2749_5091813	1A	92574191	0.00092958	3.04
FLA_T1	GLM	wsnp_Ex_c66106_64268316	1A	108693267	0.00092958	3.04
FLA_T1	GLM	Kukri_rep_c101218_200	1A	108760658	0.00058129	3.24
FLA_T1	GLM	wsnp_Ku_rep_c68419_67400635	1A	112036549	0.00092958	3.04
FLA_T1	GLM	Ex_c801_820	1A	112827164	0.00092958	3.04
FLA_T1	GLM	wsnp_Ex_c8162_13799067	1A	113878328	0.00092958	3.04
FLA_T1	GLM	IAAV2342	1A	115359114	0.00092958	3.04
FLA_T1	GLM	BS00028874_51	1A	117808227	0.00058129	3.24
FLA_T1	GLM	Excalibur_c35312_109	1A	129999811	0.00092958	3.04
FLA_T1	GLM	wsnp_Ku_c3468_6420199	1A	132485926	0.00092958	3.04
FLA_T1	GLM	RAC875_c21620_1359	1A	132784465	0.00092958	3.04
FLA_T1	GLM	wsnp_Ex_rep_c68183_66958099	1A	132785472	0.00092958	3.04
FLA_T1	GLM	RAC875_rep_c70404_755	1A	132785614	0.00092958	3.04
FLA_T1	GLM	IAAV8664	1A	134002044	0.00092958	3.04
FLA_T1	GLM	wsnp_Ku_rep_c104517_90964418	1A	135509522	0.00092958	3.04
FLA_T1	GLM	wsnp_Ex_rep_c105244_89727546	1A	136959259	0.00092958	3.04
FLA_T1	GLM	BS00030644_51	1A	138228904	0.00013155	3.89
FLA_T1	GLM	BS00066308_51	1A	147908325	0.00058129	3.24
FLA_T1	GLM	BS00003813_51	1A	150894834	0.00092958	3.04
FLA_T1	GLM	wsnp_Ex_c2389_4479352	1A	155079339	0.00092958	3.04
FLA_T1	GLM	wsnp_Ex_c2389_4479047	1A	155080158	0.00058129	3.24
FLA_T1	GLM	wsnp_Ex_c2389_4477621	1A	155081789	0.00092958	3.04
FLA_T1	GLM	BS00033760_51	1A	157831818	0.00058129	3.24
FLA_T1	GLM	wsnp_Ex_rep_c104050_88861052	1A	163393937	0.00092958	3.04
FLA_T1	GLM	RAC875_c2021_1417	1A	166322347	0.00092958	3.04
FLA_T1	GLM	RAC875_c2313_410	1A	175173990	0.00092958	3.04
FLA_T1	GLM	wsnp_Ex_c41237_48104282	1A	176457891	0.00058129	3.24
FLA_T1	GLM	wsnp_Ex_c26800_36025663	1A	177480056	0.00092958	3.04
FLA_T1	GLM	BS00076538_51	1A	180412596	0.00012644	3.9
FLA_T1	GLM	Ku_c2898_1284	1A	182069500	0.0001373	3.87
FLA_T1	GLM	Ex_c3799_2429	1A	182176198	0.0004995	3.31
FLA_T1	GLM	wsnp_Ex_c13724_21535046	1A	185141694	0.00012644	3.9
FLA_T1	GLM	GENE-2795_120	1A	186989962	0.00092958	3.04
FLA_T1	GLM	wsnp_CAP11_c3968_1874257	1A	201744663	0.00092958	3.04
FLA_T1	GLM	IAAV3998	1A	201744863	0.00092958	3.04
FLA_T1	GLM	wsnp_Ex_c17684_26426672	1A	201746360	0.00092958	3.04
FLA_T1	GLM	wsnp_Ex_c15852_24239968	1A	203817383	0.00092958	3.04
FLA_T1	GLM	wsnp_Ra_c26956_36503468	1A	208208286	0.00092958	3.04
FLA_T1	GLM	wsnp_Ex_rep_c66875_65276404	1A	208209736	0.00058129	3.24
FLA_T1	GLM	Kukri_rep_c81206_235	1A	208681924	0.00010855	3.97
FLA_T1	GLM	RAC875_c25442_231	1A	216415093	0.00092958	3.04
FLA_T1	GLM	wsnp_CAP12_c2645_1267978	1A	224805844	0.00092958	3.04

FLA_T1	GLM	RAC875_c7305_75	1A	232606133	0.00092958	3.04
FLA_T1	GLM	wsnp_Ex_c15722_24074399	1A	233924247	0.00092958	3.04
FLA_T1	GLM	BS00077815_51	1A	238653648	0.00092958	3.04
FLA_T1	GLM	wsnp_Ex_c6826_11775106	1A	238680348	0.00092958	3.04
FLA_T1	GLM	BS00063063_51	1A	240318330	0.00092958	3.04
FLA_T1	GLM	wsnp_Ex_c6278_10941843	1A	242012556	0.00092958	3.04
FLA_T1	GLM	IAAV742	1A	246129945	0.00033827	3.48
FLA_T1	GLM	wsnp_Ex_c18196_27006489	1A	249053411	0.00059993	3.23
FLA_T1	GLM	Kukri_c46608_277	1A	252624641	0.00096138	3.02
FLA_T1	GLM	BS00021895_51	1A	270007846	0.0004798	3.32
FLA_T1	GLM	BS00021730_51	1A	272313191	0.0004798	3.32
FLA_T1	GLM	BS00048887_51	1A	274785571	0.0004798	3.32
FLA_T1	GLM	GENE-0287_28	1A	276971953	0.0004798	3.32
FLA_T1	GLM	BS00065930_51	1A	289328523	0.00017593	3.76
FLA_T1	GLM	Kukri_rep_c99813_69	1A	295585339	0.00078777	3.11
FLA_T1	GLM	CAP8_c806_297	1A	297380892	0.0004798	3.32
FLA_T1	GLM	BS00085851_51	1A	309401734	0.00092958	3.04
FLA_T1	GLM	BS00064679_51	1A	311806302	0.00038626	3.42
FLA_T1	GLM	Excalibur_rep_c110450_286	1A	315835232	0.00080889	3.1
FLA_T1	MLM	Kukri_rep_c81206_235	1A	208681924	0.00071156	3.15
FLA_T1	MLM	BS00065930_51	1A	289328523	0.00061466	3.22
FLA_T2	GLM	RAC875_c63359_1446	1A	2222816	0.00054687	3.27
GpS_C	FarmCPU	wsnp_Ku_c23012_32893918	1A	516373127	0.00061084	3.22
GpS_C	FarmCPU	BS00034899_51	1A	548941277	0.00083913	3.08
GpS_C	FarmCPU	CAP12_c6629_301	1A	564279293	0.00089727	3.05
GpS_C	GLM	wsnp_Ku_c23012_32893918	1A	516373127	0.00074348	3.13
GpS_C	GLM	wsnp_CAP11_c29_68486	1A	548563957	0.00061888	3.21
GpS_C	GLM	BS00034899_51	1A	548941277	0.00022283	3.66
GpS_C	GLM	CAP12_c6629_301	1A	564279293	0.00054591	3.27
GpS_T1	FarmCPU	BS00022701_51	1A	12098792	0.0007365	3.14
GpS_T2	FarmCPU	Kukri_c14635_73	1A	551315378	0.00017916	3.75
GpS_T2	GLM	Kukri_c14635_73	1A	551315378	0.00025826	3.59
GpS_T2	MLM	Kukri_c14635_73	1A	551315378	0.00054569	3.27
NDVI_C	FarmCPU	IACX11112	1A	251590348	0.00036669	3.44
NDVI_C	FarmCPU	Tdurum_contig5560_193	1A	593287138	6.91E-05	4.17
NDVI_C	GLM	IACX11112	1A	251590348	0.00011131	3.96
NDVI_C	GLM	Tdurum_contig5560_193	1A	593287138	3.24E-05	4.49
NDVI_C	MLM	IACX11112	1A	251590348	0.00083956	3.08
NDVI_C	MLM	Tdurum_contig5560_193	1A	593287138	0.00030993	3.51
NDVI_T1	GLM	BobWhite_c6664_644	1A	574935621	0.00039849	3.4
NDVI_T2	FarmCPU	tplb0030a05_2386	1A	569450220	0.00054961	3.26
NDVI_T2	GLM	tplb0030a05_2386	1A	569450220	0.00091738	3.04
PH_C	FarmCPU	Tdurum_contig60037_441	1A	23966387	0.00086234	3.07
PH_C	FarmCPU	wsnp_Ku_c4413_8008008	1A	377427767	0.00095511	3.02
PH_C	FarmCPU	BS00110766_51	1A	377614488	0.00094581	3.03
PH_C	FarmCPU	Kukri_c44895_88	1A	564749691	8.31E-05	4.09

PH_C	GLM	Tdurum_contig60037_441	1A	23966387	0.00017441	3.76
PH_C	GLM	wsnp_Ku_c4413_8008008	1A	377427767	0.0009961	3.01
PH_C	GLM	BS00070580_51	1A	544170185	0.00091749	3.04
PH_C	GLM	RFL_Contig399_976	1A	549420559	0.00046138	3.34
PH_C	GLM	BS00072408_51	1A	558536578	0.00094441	3.03
PH_C	GLM	Kukri_c44895_88	1A	564749691	1.83E-05	4.74
PH_C	GLM	BobWhite_c6820_199	1A	571580102	0.00065546	3.19
PH_C	GLM	wsnp_Ex_c3264_6017750	1A	571580252	0.00024197	3.62
PH_C	MLM	Kukri_c44895_88	1A	564749691	0.00039401	3.41
PH_T1	FarmCPU	TA001286-0611-w	1A	3777195	0.00099485	3.01
PH_T1	FarmCPU	RAC875_c29598_147	1A	20094011	0.00064301	3.2
PH_T1	GLM	RAC875_c29598_147	1A	20094011	0.00061156	3.22
PH_T2	FarmCPU	Ex_c42595_2332	1A	445880641	0.00060248	3.23
PH_T2	GLM	Ex_c42595_2332	1A	445880641	0.00070456	3.16
T.P_C	FarmCPU	RAC875_c11899_366	1A	58704679	0.00025493	3.6
T.P_C	FarmCPU	IACX6344	1A	92567883	0.00040035	3.4
T.P_C	GLM	RAC875_c11899_366	1A	58704679	0.00053334	3.28
T.P_C	MLM	RAC875_c11899_366	1A	58704679	0.00070509	3.16
T.P_C	MLM	IACX6344	1A	92567883	0.00098468	3.01
HI_C	FarmCPU	wsnp_Ex_c7965_13520238	1A	12369332	0.00050557	3.3
HI_C	GLM	wsnp_Ex_c7965_13520238	1A	12369332	0.00046618	3.34
HI_T1	FarmCPU	wsnp_Ex_c12254_19574891	1A	3386505	3.29E-05	4.49
HI_T1	GLM	RAC875_c46269_387	1A	57768485	0.00011572	3.94
HI_T1	GLM	wsnp_Ex_c2389_4478587	1A	155080736	0.00011572	3.94
HI_T1	GLM	wsnp_Ra_rep_c105422_89367749	1A	223938286	0.00091892	3.04
HI_T1	MLM	RAC875_c46269_387	1A	57768485	0.00032523	3.49
HI_T1	MLM	wsnp_Ex_c2389_4478587	1A	155080736	0.00032523	3.49
NAE_T2	FarmCPU	Tdurum_contig11756_458	1A	20094515	0.00053328	3.28
NAE_T2	GLM	Tdurum_contig11756_458	1A	20094515	0.00060481	3.22
BM_C	FarmCPU	Kukri_c8235_371	1B	560494382	4.9371E-05	4.31
BM_C	FarmCPU	Excalibur_c6892_274	1B	555638118	0.00034475	3.47
BM_C	FarmCPU	RAC875_c275_229	1B	571437063	0.00034475	3.47
BM_C	FarmCPU	wsnp_Ku_c8235_14030979	1B	560494393	0.0003964	3.41
BM_C	FarmCPU	Kukri_c45852_78	1B	562375788	0.00040167	3.4
BM_C	FarmCPU	BS00081395_51	1B	555933481	0.00051171	3.3
BM_C	FarmCPU	RAC875_c24895_311	1B	561507383	0.0005556	3.26
BM_C	FarmCPU	Kukri_rep_c113407_250	1B	561507652	0.0005556	3.26
BM_C	FarmCPU	tplb0048b10_1365	1B	559966786	0.0005556	3.26
BM_C	FarmCPU	wsnp_Ex_rep_c66389_64589189	1B	561703732	0.0005556	3.26
BM_C	FarmCPU	wsnp_Ex_rep_c66389_64588992	1B	561704620	0.0005556	3.26
BM_C	FarmCPU	Ku_c106533_550	1B	563675285	0.0005556	3.26
BM_C	FarmCPU	BS00089563_51	1B	560252448	0.00087003	3.07
BM_C	GLM	Excalibur_c6892_274	1B	555638118	0.00043902	3.36
BM_C	GLM	BS00081395_51	1B	555933481	0.000646	3.19
BM_C	GLM	tplb0048b10_1365	1B	559966786	0.00060577	3.22
BM_C	GLM	BS00089563_51	1B	560252448	0.00086289	3.07

BM_C	GLM	Kukri_c8235_371	1B	560494382	5.53E-05	4.26
BM_C	GLM	wsnp_Ku_c8235_14030979	1B	560494393	0.00041	3.39
BM_C	GLM	RAC875_c24895_311	1B	561507383	0.00060577	3.22
BM_C	GLM	Kukri_rep_c113407_250	1B	561507652	0.00060577	3.22
BM_C	GLM	wsnp_Ex_rep_c66389_64589189	1B	561703732	0.00060577	3.22
BM_C	GLM	wsnp_Ex_rep_c66389_64588992	1B	561704620	0.00060577	3.22
BM_C	GLM	Kukri_c45852_78	1B	562375788	0.00048645	3.32
BM_C	GLM	Ku_c106533_550	1B	563675285	0.00060577	3.22
BM_C	GLM	RAC875_c275_229	1B	571437063	0.00043902	3.36
BM_C	MLM	Excalibur_c6892_274	1B	555638118	0.00087538	3.06
BM_C	MLM	Kukri_c8235_371	1B	560494382	0.00023899	3.63
BM_C	MLM	RAC875_c275_229	1B	571437063	0.00087538	3.06
BM_T1	GLM	RFL_Contig2160_524	1B	46884268	0.00079308	3.11
CHL_C	FarmCPU	Excalibur_rep_c113987_164	1B	4346959	0.00055733	3.26
CHL_C	FarmCPU	RAC875_rep_c74067_541	1B	17251494	0.00016215	3.8
CHL_C	FarmCPU	IAAV8117	1B	17812498	0.00055733	3.26
CHL_C	FarmCPU	Tdurum_contig50667_299	1B	20588113	0.00049252	3.31
CHL_C	FarmCPU	tplb0055p02_1074	1B	24932334	0.00055733	3.26
CHL_C	FarmCPU	wsnp_BE405834B_Ta_2_3	1B	28765583	0.00055733	3.26
CHL_C	FarmCPU	BS00022590_51	1B	38911372	0.00065433	3.19
CHL_C	FarmCPU	BS00064929_51	1B	42014807	0.00055733	3.26
CHL_C	FarmCPU	BS00087787_51	1B	50778549	0.00055733	3.26
CHL_C	FarmCPU	BS00087133_51	1B	52900618	0.00055733	3.26
CHL_C	FarmCPU	BS00022604_51	1B	52900648	0.00055733	3.26
CHL_C	FarmCPU	IACX18625	1B	57712478	0.00055733	3.26
CHL_C	FarmCPU	RAC875_c99469_172	1B	64236462	0.00055733	3.26
CHL_C	FarmCPU	Excalibur_c19341_673	1B	69539089	0.00055733	3.26
CHL_C	FarmCPU	BS00023056_51	1B	69542830	0.00055733	3.26
CHL_C	FarmCPU	IACX13974	1B	69826757	0.00055733	3.26
CHL_C	FarmCPU	BS00083533_51	1B	69906151	0.00055733	3.26
CHL_C	FarmCPU	Kukri_c7129_227	1B	69907136	0.00055733	3.26
CHL_C	FarmCPU	Tdurum_contig25732_112	1B	70192585	0.00055733	3.26
CHL_C	FarmCPU	BS00022745_51	1B	70711205	0.00055733	3.26
CHL_C	FarmCPU	BS00057730_51	1B	82310917	0.00055733	3.26
CHL_C	FarmCPU	Tdurum_contig29169_289	1B	86792760	0.00055733	3.26
CHL_C	FarmCPU	RAC875_c98832_52	1B	86792815	0.00055733	3.26
CHL_C	FarmCPU	BS00004453_51	1B	91557656	0.00055733	3.26
CHL_C	FarmCPU	Tdurum_contig75545_71	1B	92310552	0.00098837	3.01
CHL_C	FarmCPU	tplb0038o14_241	1B	94956280	0.00055733	3.26
CHL_C	FarmCPU	Kukri_c43552_443	1B	95736705	0.00055733	3.26
CHL_C	FarmCPU	Kukri_c43552_238	1B	95736910	0.00055733	3.26
CHL_C	FarmCPU	BS00101816_51	1B	96593418	0.00023566	3.63
CHL_C	FarmCPU	RAC875_rep_c108757_136	1B	98722083	0.00055733	3.26
CHL_C	FarmCPU	BS00069316_51	1B	98904315	0.00055733	3.26
CHL_C	FarmCPU	CAP11_c1320_264	1B	101590765	0.00023513	3.63
CHL_C	FarmCPU	IACX6397	1B	104721235	0.00055733	3.26

CHL_C	FarmCPU	GENE-0403_301	1B	109728575	0.00055733	3.26
CHL_C	FarmCPU	wsnp_Ex_c11976_19193992	1B	109729963	0.00055733	3.26
CHL_C	FarmCPU	Tdurum_contig19251_352	1B	112864418	0.00055733	3.26
CHL_C	FarmCPU	Tdurum_contig19251_515	1B	112864581	0.00055733	3.26
CHL_C	FarmCPU	CAP8_c311_448	1B	115283669	0.00055733	3.26
CHL_C	FarmCPU	BS00069054_51	1B	115285489	0.00055733	3.26
CHL_C	FarmCPU	Ku_c3998_1400	1B	116977449	0.00055733	3.26
CHL_C	FarmCPU	IACX20344	1B	117183520	0.00055733	3.26
CHL_C	FarmCPU	RAC875_c455_1400	1B	117188037	0.00055733	3.26
CHL_C	FarmCPU	BS00093946_51	1B	119767719	0.00055733	3.26
CHL_C	FarmCPU	RAC875_c51813_182	1B	133113569	0.00055733	3.26
CHL_C	FarmCPU	BS00073034_51	1B	136645710	0.00044793	3.35
CHL_C	FarmCPU	GENE-0542_613	1B	142523514	0.00055733	3.26
CHL_C	FarmCPU	GENE-4608_406	1B	144274345	0.00042103	3.38
CHL_C	FarmCPU	Tdurum_contig31387_156	1B	151461413	0.00055733	3.26
CHL_C	FarmCPU	BS00087138_51	1B	151472739	0.00055733	3.26
CHL_C	FarmCPU	BS00004465_51	1B	155407460	0.00055733	3.26
CHL_C	FarmCPU	BS00064327_51	1B	161541537	0.00055733	3.26
CHL_C	FarmCPU	GENE-0456_190	1B	162811401	0.00055733	3.26
CHL_C	FarmCPU	BS00066971_51	1B	163585426	0.00055733	3.26
CHL_C	FarmCPU	Ra_c6693_1297	1B	163662718	0.00055733	3.26
CHL_C	FarmCPU	BS00106579_51	1B	166078425	0.00055733	3.26
CHL_C	FarmCPU	BS00026541_51	1B	169642380	0.00055733	3.26
CHL_C	FarmCPU	BS00081963_51	1B	169662066	0.00055733	3.26
CHL_C	FarmCPU	Kukri_rep_c115647_349	1B	171044974	0.00055733	3.26
CHL_C	FarmCPU	RAC875_s117310_106	1B	183878165	0.00055733	3.26
CHL_C	FarmCPU	BS00095751_51	1B	188262333	0.00055733	3.26
CHL_C	FarmCPU	BS00015169_51	1B	189715586	0.00055733	3.26
CHL_C	FarmCPU	RAC875_rep_c115865_721	1B	205419440	0.00055733	3.26
CHL_C	FarmCPU	Tdurum_contig27840_304	1B	209133171	0.00055733	3.26
CHL_C	FarmCPU	RAC875_c30593_58	1B	211312727	0.00055733	3.26
CHL_C	FarmCPU	BobWhite_c4147_1429	1B	236958535	0.00055733	3.26
CHL_C	GLM	BS00111170_51	1B	5694698	0.00033002	3.49
CHL_C	GLM	BS00030768_51	1B	5855038	0.00068973	3.17
CHL_C	GLM	Ra_c78638_309	1B	5990557	0.00013997	3.86
CHL_C	GLM	RAC875_rep_c74067_541	1B	17251494	0.00036142	3.45
CHL_C	GLM	Tdurum_contig50667_299	1B	20588113	0.00087495	3.06
CHL_C	GLM	BS00022590_51	1B	38911372	0.00065439	3.19
CHL_C	GLM	BS00101816_51	1B	96593418	0.00042344	3.38
CHL_C	GLM	CAP11_c1320_264	1B	101590765	0.00040851	3.39
CHL_C	GLM	BS00073034_51	1B	136645710	0.00061597	3.22
CHL_C	GLM	GENE-4608_406	1B	144274345	0.00072169	3.15
CHL_T1	MLM	Excalibur_rep_c101787_89	1B	608996477	0.00015688	3.81
CHL_T1	MLM	BS00062810_51	1B	610149405	0.00047142	3.33
CHL_T1	FarmCPU	BS00021667_51	1B	8830567	0.0005933	3.23
CHL_T1	FarmCPU	Excalibur_rep_c101787_89	1B	608996477	2.29E-05	4.64

CHL_T1	FarmCPU	BS00062810_51	1B	610149405	0.00011443	3.95
CHL_T1	FarmCPU	Excalibur_rep_c96924_118	1B	623712982	0.00063026	3.21
CHL_T1	GLM	BS00021667_51	1B	8830567	0.00014883	3.83
CHL_T1	GLM	Excalibur_rep_c101787_89	1B	608996477	0.00011869	3.93
CHL_T1	GLM	BS00062810_51	1B	610149405	0.00044416	3.36
FLA_C	FarmCPU	IAAV1833	1B	20587566	0.00091125	3.05
FLA_C	FarmCPU	IAAV8952	1B	49880968	0.00057601	3.24
FLA_C	FarmCPU	Kukri_c11389_232	1B	619683503	0.00025326	3.6
FLA_C	FarmCPU	IACX8117	1B	621270298	0.00025326	3.6
FLA_C	FarmCPU	BS00022530_51	1B	622314014	0.00025326	3.6
FLA_C	GLM	RAC875_c50835_124	1B	20589434	0.00033979	3.47
FLA_C	GLM	Kukri_c11389_232	1B	619683503	0.00041902	3.38
FLA_C	GLM	IACX8117	1B	621270298	0.00041902	3.38
FLA_C	GLM	BS00022530_51	1B	622314014	0.00041902	3.38
FLA_C	MLM	Kukri_c11389_232	1B	619683503	0.00094127	3.03
FLA_C	MLM	IACX8117	1B	621270298	0.00094127	3.03
FLA_C	MLM	BS00022530_51	1B	622314014	0.00094127	3.03
FLA_T1	FarmCPU	GENE-0379_108	1B	286612009	0.00081078	3.1
FLA_T1	FarmCPU	BS00073603_51	1B	333208215	0.00028774	3.55
FLA_T1	FarmCPU	wsnp_BF200640B_Ta_2_1	1B	627946628	0.00028155	3.56
FLA_T1	GLM	RAC875_c40444_84	1B	17251393	0.00098727	3.01
FLA_T1	GLM	GENE-0379_108	1B	286612009	0.0004798	3.32
FLA_T1	GLM	BS00073603_51	1B	333208215	0.00024462	3.62
FLA_T1	GLM	IACX8300	1B	341931593	0.00092958	3.04
FLA_T1	GLM	wsnp_BF200640B_Ta_2_1	1B	627946628	0.00018938	3.73
FLA_T1	MLM	BS00073603_51	1B	333208215	0.00077075	3.12
FLA_T1	MLM	wsnp_BF200640B_Ta_2_1	1B	627946628	0.00075854	3.13
FLA_T2	FarmCPU	Tdurum_contig63370_207	1B	676079638	0.00071856	3.15
FLA_T2	FarmCPU	RAC875_c24317_1351	1B	676081849	0.00031335	3.51
FLA_T2	GLM	RAC875_rep_c113481_415	1B	666917911	0.00025957	3.59
FLA_T2	GLM	Tdurum_contig63370_207	1B	676079638	0.00069413	3.16
FLA_T2	GLM	RAC875_c24317_1351	1B	676081849	0.00042938	3.37
FLA_T2	MLM	RAC875_c24317_1351	1B	676081849	0.00082079	3.09
GpS_C	GLM	Excalibur_c7684_54	1B	660531534	0.00078393	3.11
GpS_C	GLM	BS00104270_51	1B	688232318	0.00084419	3.08
GY_C	FarmCPU	CAP7_rep_c6866_212	1B	172383664	8.66E-05	4.07
GY_C	FarmCPU	wsnp_Ex_c22439_31632880	1B	563027914	0.00021258	3.68
GY_C	FarmCPU	JD_c107_683	1B	563675996	5.70E-06	5.25
GY_C	FarmCPU	Kukri_c25961_108	1B	575863800	0.00090769	3.05
GY_C	FarmCPU	Kukri_c25961_166	1B	575863858	0.00042619	3.38
GY_C	FarmCPU	IACX11274	1B	576024228	0.00021944	3.66
GY_C	FarmCPU	Kukri_c8143_355	1B	581201878	0.00015873	3.8
GY_C	FarmCPU	BS00022551_51	1B	583446285	4.35E-06	5.37
GY_C	FarmCPU	Ku_c1932_1583	1B	584156264	4.00E-05	4.4
GY_C	GLM	CAP7_rep_c6866_212	1B	172383664	0.00018531	3.74
GY_C	GLM	wsnp_Ex_c22439_31632880	1B	563027914	0.00046737	3.34

GY_C	GLM	JD_c107_683	1B	563675996	1.08E-05	4.97
GY_C	GLM	Kukri_c25961_166	1B	575863858	0.00036347	3.44
GY_C	GLM	IACX11274	1B	576024228	0.00027093	3.57
GY_C	GLM	Kukri_c8143_355	1B	581201878	0.00020512	3.69
GY_C	GLM	BS00022551_51	1B	583446285	6.11E-06	5.22
GY_C	GLM	Ku_c1932_1583	1B	584156264	5.34E-05	4.28
GY_C	MLM	CAP7_rep_c6866_212	1B	172383664	0.00089406	3.05
GY_C	MLM	JD_c107_683	1B	563675996	0.00015392	3.82
GY_C	MLM	BS00022551_51	1B	583446285	6.18E-05	4.21
GY_C	MLM	Ku_c1932_1583	1B	584156264	0.00031354	3.51
GY_T2	FarmCPU	BS00110276_51	1B	2394933	0.00024722	3.61
NDVI_T2	FarmCPU	BS00107675_51	1B	634412953	0.00021441	3.67
NDVI_T2	GLM	BS00107675_51	1B	634412953	0.00033247	3.48
PH_C	FarmCPU	BS00051105_51	1B	99594508	0.00065486	3.19
PH_C	FarmCPU	wsnp_Ex_c52474_56060204	1B	450610959	0.00041783	3.38
PH_C	FarmCPU	tplb0043a07_880	1B	637622677	0.00040355	3.4
PH_C	GLM	RAC875_rep_c106876_558	1B	573572299	0.0006234	3.21
PH_T1	FarmCPU	GENE-0815_140	1B	15142189	0.00067043	3.18
PH_T1	FarmCPU	wsnp_JD_c6331_7499060	1B	548967983	0.00050961	3.3
PH_T1	GLM	GENE-0815_140	1B	15142189	0.0006601	3.19
PH_T1	GLM	wsnp_JD_c6331_7499060	1B	548967983	0.0003988	3.4
HI_C	FarmCPU	BS00108058_51	1B	15439329	0.00047279	3.33
HI_C	FarmCPU	Excalibur_c60931_1260	1B	563030480	9.96E-05	4.01
HI_C	GLM	BS00108058_51	1B	15439329	0.00034362	3.47
HI_C	GLM	Excalibur_c60931_1260	1B	563030480	0.00017656	3.76
HI_C	MLM	Excalibur_c60931_1260	1B	563030480	0.00035916	3.45
HI_T1	GLM	BS00071333_51	1B	4094859	0.00053024	3.28
HI_T1	MLM	BS00071333_51	1B	4094859	0.00082272	3.09
NAE_T1	FarmCPU	BS00022551_51	1B	583446285	0.00018542	3.74
NAE_T1	FarmCPU	Ku_c1932_1583	1B	584156264	0.0003822	3.42
NAE_T1	GLM	BS00022551_51	1B	583446285	0.00014119	3.86
NAE_T1	GLM	Ku_c1932_1583	1B	584156264	0.00054506	3.27
NAE_T1	MLM	BS00022551_51	1B	583446285	0.00055937	3.26
NAE_T1	MLM	Ku_c1932_1583	1B	584156264	0.00095126	3.03
BM_C	FarmCPU	Kukri_c36329_526	1D	427618868	0.00034475	3.47
BM_C	FarmCPU	BS00089031_51	1D	424737017	0.00090528	3.05
BM_C	GLM	Kukri_c36329_526	1D	427618868	0.00043902	3.36
BM_C	MLM	Kukri_c36329_526	1D	427618868	0.00087538	3.06
BM_T2	FarmCPU	BS00108591_51	1D	486758137	0.00091436	3.04
BM_T2	GLM	Kukri_c82086_387	1D	455802372	0.00046527	3.34
BM_T2	GLM	BS00108591_51	1D	486758137	0.00056175	3.26
CHL_C	FarmCPU	IACX6862	1D	19185651	0.00055733	3.26
CHL_C	FarmCPU	GENE-3075_364	1D	31429780	0.00055733	3.26
CHL_C	FarmCPU	RAC875_c2444_101	1D	31430684	0.00055733	3.26
CHL_C	FarmCPU	IAAV960	1D	47332508	0.00055733	3.26
CHL_C	FarmCPU	IAAV4825	1D	63374079	0.00055733	3.26

CHL_C	FarmCPU	BS00058711_51	1D	63637747	0.00055733	3.26
CHL_C	FarmCPU	RAC875_c90431_188	1D	77392348	0.00055733	3.26
CHL_C	FarmCPU	Kukri_c7936_630	1D	106236859	0.00065433	3.19
CHL_C	FarmCPU	Excalibur_c43347_322	1D	109942402	0.00055733	3.26
CHL_C	FarmCPU	GENE-0450_892	1D	118885713	0.00055733	3.26
CHL_C	FarmCPU	GENE-0339_174	1D	123104812	0.00055733	3.26
CHL_C	GLM	Kukri_c7936_630	1D	106236859	0.00065439	3.19
FLA_C	FarmCPU	Kukri_c9170_778	1D	38911104	0.00060054	3.23
FLA_C	GLM	Kukri_c9170_778	1D	38911104	0.00066056	3.19
FLA_T1	FarmCPU	Kukri_c34519_630	1D	166832499	0.00078786	3.11
FLA_T1	GLM	IAAV3422	1D	28576058	0.0005982	3.23
FLA_T1	GLM	BS00049071_51	1D	28579451	0.00088477	3.06
FLA_T1	GLM	BS00049072_51	1D	28579543	0.00088477	3.06
FLA_T1	GLM	Kukri_c34519_630	1D	166832499	0.00030989	3.51
FLA_T1	GLM	BS00009866_51	1D	236438835	0.00092958	3.04
FLA_T2	FarmCPU	RFL_Contig5090_1510	1D	41720718	0.0004934	3.31
FLA_T2	FarmCPU	wsnp_Ra_rep_c97840_85062318	1D	485557735	0.00056539	3.25
FLA_T2	FarmCPU	BobWhite_c6433_417	1D	485559318	0.00056539	3.25
FLA_T2	GLM	RFL_Contig5090_1510	1D	41720718	0.00070585	3.16
FLA_T2	GLM	wsnp_Ra_rep_c97840_85062318	1D	485557735	0.00063324	3.2
FLA_T2	GLM	BobWhite_c6433_417	1D	485559318	0.00063324	3.2
GY_C	FarmCPU	BS00089031_51	1D	424737017	0.00041482	3.39
GY_C	FarmCPU	GENE-0487_795	1D	426416291	4.49E-05	4.35
GY_C	GLM	BS00089031_51	1D	424737017	0.00078829	3.11
GY_C	GLM	GENE-0487_795	1D	426416291	5.11E-05	4.3
GY_C	MLM	GENE-0487_795	1D	426416291	0.0004959	3.31
GY_T2	FarmCPU	Excalibur_rep_c111074_76	1D	2094286	0.00027314	3.57
GY_T2	GLM	wsnp_Ku_c7822_13408189	1D	2091240	0.00029447	3.54
GY_T2	GLM	RAC875_c10387_685	1D	11399886	0.00025467	3.6
GY_T2	MLM	wsnp_Ku_c7822_13408189	1D	2091240	0.00084231	3.08
GY_T2	MLM	Excalibur_rep_c111074_76	1D	2094286	0.0007181	3.15
NDVI_C	GLM	wsnp_Ex_c1358_2602235	1D	8606537	0.00036607	3.44
PH_C	FarmCPU	Ra_c7324_1464	1D	462488065	0.00040355	3.4
PH_C	GLM	BobWhite_c39092_629	1D	470894146	0.00033167	3.48
PH_C	GLM	RAC875_c36980_161	1D	472520824	0.00022641	3.65
HI_C	FarmCPU	Excalibur_c45969_370	1D	11180056	0.00020822	3.69
HI_C	GLM	Excalibur_c45969_370	1D	11180056	0.0001911	3.72
HI_C	MLM	Excalibur_c45969_370	1D	11180056	0.00060833	3.22
NAE_T2	FarmCPU	RAC875_rep_c69721_835	1D	101942866	1.51E-05	4.83
NAE_T2	FarmCPU	wsnp_BE424100D_Ta_1_1	1D	229066958	0.00025321	3.6
NAE_T2	GLM	RAC875_rep_c69721_835	1D	101942866	1.81E-05	4.75
NAE_T2	GLM	wsnp_BE424100D_Ta_1_1	1D	229066958	0.00017124	3.77
NAE_T2	MLM	RAC875_rep_c69721_835	1D	101942866	0.00010768	3.97
NAE_T2	MLM	wsnp_BE424100D_Ta_1_1	1D	229066958	0.00078611	3.11
BM_C	FarmCPU	RAC875_c51459_311	2A	779671024	0.00030306	3.52
BM_C	FarmCPU	BS00049816_51	2A	778597017	0.00086936	3.07

BM_C	GLM	Jagger_c9722_251	2A	753965758	0.00053564	3.28
BM_C	GLM	RAC875_c51459_311	2A	779671024	0.0004039	3.4
BM_C	MLM	RAC875_c51459_311	2A	779671024	0.00090234	3.05
CHL_T1	FarmCPU	Excalibur_rep_c104620_183	2A	740360656	0.00047607	3.33
FLA_C	FarmCPU	IAAV5232	2A	760565482	0.00055464	3.26
FLA_C	FarmCPU	Tdurum_contig42282_10323	2A	760569195	0.00060671	3.22
FLA_C	GLM	IAAV5232	2A	760565482	0.00085093	3.08
FLA_C	GLM	Tdurum_contig42282_10323	2A	760569195	0.00082909	3.09
FLA_T1	FarmCPU	BobWhite_c19433_185	2A	30866270	0.00057671	3.24
FLA_T1	GLM	BobWhite_c19433_185	2A	30866270	0.00069543	3.16
GpS_C	GLM	Kukri_c33374_1048	2A	4790334	0.0003097	3.51
GpS_T1	FarmCPU	Tdurum_contig45580_2786	2A	93882600	0.00081964	3.09
GpS_T1	FarmCPU	Excalibur_c21269_176	2A	93926917	5.71E-05	4.25
GpS_T1	FarmCPU	Excalibur_c21872_135	2A	101382979	0.00010295	3.99
GpS_T1	FarmCPU	Kukri_c44442_274	2A	123548428	6.09E-05	4.22
GpS_T1	GLM	Excalibur_c21269_176	2A	93926917	0.00028123	3.56
GpS_T1	GLM	Excalibur_c21872_135	2A	101382979	0.0003579	3.45
GpS_T1	GLM	Kukri_c44442_274	2A	123548428	0.0002503	3.61
GpS_T1	MLM	Excalibur_c21269_176	2A	93926917	0.00031236	3.51
GpS_T1	MLM	Excalibur_c21872_135	2A	101382979	0.00042519	3.38
GpS_T1	MLM	Kukri_c44442_274	2A	123548428	0.00028899	3.54
GpS_T2	FarmCPU	wsnp_Ex_c15681_24016359	2A	739875850	0.00047802	3.33
GpS_T2	GLM	wsnp_Ex_c15681_24016359	2A	739875850	0.00057082	3.25
GY_C	FarmCPU	BS00065110_51	2A	31088889	0.00027877	3.56
GY_C	FarmCPU	BS00087932_51	2A	779673613	0.00074055	3.14
GY_C	FarmCPU	BS00087929_51	2A	779673657	0.00059367	3.23
GY_C	GLM	wsnp_Ku_c33374_42877546	2A	4789998	0.00058171	3.24
GY_C	GLM	BS00065110_51	2A	31088889	0.00042624	3.38
GY_C	GLM	wsnp_CAP11_c1737_946813	2A	770018214	0.00098491	3.01
GY_C	GLM	BS00049932_51	2A	771506951	0.00031194	3.51
GY_T2	GLM	wsnp_Ku_c10302_17079851	2A	50055471	0.00061249	3.22
NDVI_C	FarmCPU	Tdurum_contig66015_346	2A	58394903	0.00084718	3.08
NDVI_T1	FarmCPU	Kukri_rep_c72412_856	2A	43137023	0.00056037	3.26
NDVI_T1	GLM	Kukri_rep_c72412_856	2A	43137023	0.00049597	3.31
NDVI_T2	FarmCPU	IAAV6409	2A	520562737	0.00037785	3.43
NDVI_T2	FarmCPU	wsnp_Ku_c2413_4626451	2A	526300374	0.00037785	3.43
NDVI_T2	FarmCPU	RAC875_c69068_71	2A	526300524	0.00037785	3.43
NDVI_T2	FarmCPU	RAC875_c58006_436	2A	715300357	0.0007799	3.11
NDVI_T2	GLM	IAAV6409	2A	520562737	0.00053141	3.28
NDVI_T2	GLM	wsnp_Ku_c2413_4626451	2A	526300374	0.00053141	3.28
NDVI_T2	GLM	RAC875_c69068_71	2A	526300524	0.00053141	3.28
PH_T1	GLM	wsnp_Ex_rep_c71983_70544041	2A	709836910	0.00096987	3.02
PH_T2	FarmCPU	wsnp_Ex_c42720_49228237	2A	520562637	0.00017111	3.77
PH_T2	FarmCPU	Kukri_c77188_798	2A	543183268	0.00017111	3.77
PH_T2	FarmCPU	BS00045521_51	2A	543306835	0.00017111	3.77
PH_T2	FarmCPU	BS00012126_51	2A	608608452	0.00017111	3.77

PH_T2	FarmCPU	Tdurum_contig12761_125	2A	727243449	0.00076538	3.12
PH_T2	FarmCPU	Tdurum_contig60205_806	2A	739712633	0.000391	3.41
PH_T2	FarmCPU	Kukri_c54944_116	2A	739818123	0.00017111	3.77
PH_T2	FarmCPU	Excalibur_c42110_210	2A	744283347	0.00092264	3.04
PH_T2	FarmCPU	BS00024506_51	2A	762762677	0.00017111	3.77
PH_T2	GLM	wsnp_Ex_c42720_49228237	2A	520562637	0.0002268	3.65
PH_T2	GLM	Kukri_c77188_798	2A	543183268	0.0002268	3.65
PH_T2	GLM	BS00045521_51	2A	543306835	0.0002268	3.65
PH_T2	GLM	BS00012126_51	2A	608608452	0.0002268	3.65
PH_T2	GLM	Tdurum_contig12761_125	2A	727243449	0.00081589	3.09
PH_T2	GLM	Tdurum_contig60205_806	2A	739712633	0.00044896	3.35
PH_T2	GLM	Kukri_c54944_116	2A	739818123	0.0002268	3.65
PH_T2	GLM	BS00024506_51	2A	762762677	0.0002268	3.65
PH_T2	MLM	wsnp_Ex_c42720_49228237	2A	520562637	0.00061337	3.22
PH_T2	MLM	Kukri_c77188_798	2A	543183268	0.00061337	3.22
PH_T2	MLM	BS00045521_51	2A	543306835	0.00061337	3.22
PH_T2	MLM	BS00012126_51	2A	608608452	0.00061337	3.22
PH_T2	MLM	Tdurum_contig60205_806	2A	739712633	0.00085946	3.07
PH_T2	MLM	Kukri_c54944_116	2A	739818123	0.00061337	3.22
PH_T2	MLM	BS00024506_51	2A	762762677	0.00061337	3.22
T.P_C	FarmCPU	Tdurum_contig50824_58	2A	550536090	0.00016055	3.8
T.P_C	FarmCPU	CAP7_c2791_231	2A	551720266	0.00016055	3.8
T.P_C	GLM	Tdurum_contig50824_58	2A	550536090	0.00019915	3.71
T.P_C	GLM	CAP7_c2791_231	2A	551720266	0.00019915	3.71
T.P_C	MLM	Tdurum_contig50824_58	2A	550536090	0.00050421	3.3
T.P_C	MLM	CAP7_c2791_231	2A	551720266	0.00050421	3.3
HI_C	FarmCPU	GENE-0910_153	2A	679887445	0.00098276	3.01
HI_T1	GLM	Tdurum_contig560_297	2A	72381618	0.00094564	3.03
HI_T1	GLM	IAAV3800	2A	758396016	0.00059707	3.23
NAE_T2	FarmCPU	wsnp_Ku_c33374_42877546	2A	4789998	0.00078158	3.11
NAE_T2	GLM	wsnp_Ku_c33374_42877546	2A	4789998	0.00083118	3.09
BM_C	FarmCPU	IAAV6032	2B	786229451	0.00010458	3.99
BM_C	FarmCPU	BS00080318_51	2B	763842605	0.0003379	3.48
BM_C	FarmCPU	Kukri_c26697_366	2B	776976026	0.00048624	3.32
BM_C	FarmCPU	Excalibur_c73027_267	2B	762518780	0.00056266	3.25
BM_C	FarmCPU	wsnp_Ex_c34419_42734849	2B	752490657	0.00094423	3.03
BM_C	GLM	wsnp_Ex_c34419_42734849	2B	752490657	0.00029614	3.53
BM_C	GLM	Excalibur_c73027_267	2B	762518780	0.00078602	3.11
BM_C	GLM	BS00080318_51	2B	763842605	0.00032997	3.49
BM_C	GLM	Kukri_c26697_366	2B	776976026	0.00067365	3.18
BM_C	GLM	IAAV6032	2B	786229451	0.00013133	3.89
BM_C	MLM	BS00080318_51	2B	763842605	0.00089715	3.05
BM_C	MLM	IAAV6032	2B	786229451	0.00041939	3.38
CHL_C	GLM	BobWhite_c662_148	2B	152569030	0.00050912	3.3
CHL_T2	FarmCPU	GENE-1018_278	2B	17783303	0.00083836	3.08
FLA_C	FarmCPU	Kukri_c52608_142	2B	797627190	0.00095308	3.03

GpS_C	FarmCPU	IAAV8632	2B	249447079	0.00045186	3.35
GpS_C	GLM	IAAV8632	2B	249447079	0.00045701	3.35
GpS_T1	FarmCPU	RAC875_c3067_1830	2B	149841639	0.00046939	3.33
GpS_T2	FarmCPU	BS00037278_51	2B	184661726	0.00087046	3.07
GpS_T2	FarmCPU	wsnp_Ku_c2562_4879681	2B	190225573	0.00080379	3.1
GY_C	GLM	IAAV6032	2B	786229451	0.0008243	3.09
GY_T1	FarmCPU	IACX9460	2B	7108861	0.00064914	3.19
GY_T1	FarmCPU	BS00031118_51	2B	7435577	0.00056803	3.25
GY_T1	FarmCPU	BS00083763_51	2B	7442993	0.00056803	3.25
GY_T1	FarmCPU	Excalibur_c7736_537	2B	454779059	0.00056803	3.25
GY_T1	FarmCPU	Excalibur_c36184_430	2B	454780257	0.00056803	3.25
GY_T1	GLM	BS00031118_51	2B	7435577	0.00097637	3.02
GY_T1	GLM	BS00083763_51	2B	7442993	0.00097637	3.02
GY_T1	GLM	Excalibur_c7736_537	2B	454779059	0.00097637	3.02
GY_T1	GLM	Excalibur_c36184_430	2B	454780257	0.00097637	3.02
GY_T2	GLM	Tdurum_contig29563_197	2B	28339730	0.00044154	3.36
NDVI_C	FarmCPU	wsnp_Ex_c10071_16554911	2B	692463526	0.0009023	3.05
NDVI_C	FarmCPU	wsnp_Ku_c9901_16493072	2B	696679853	0.0006835	3.17
NDVI_C	FarmCPU	BS00046164_51	2B	697510323	8.28E-05	4.09
NDVI_C	FarmCPU	BS00046165_51	2B	697510334	6.62E-05	4.18
NDVI_C	FarmCPU	RAC875_c4465_549	2B	699108786	0.00056034	3.26
NDVI_C	FarmCPU	wsnp_Ex_c22271_31463467	2B	700456564	0.00086144	3.07
NDVI_C	GLM	BS00046164_51	2B	697510323	0.00017152	3.77
NDVI_C	GLM	BS00046165_51	2B	697510334	0.00016201	3.8
NDVI_C	MLM	BS00046164_51	2B	697510323	0.00042682	3.37
NDVI_C	MLM	BS00046165_51	2B	697510334	0.00036642	3.44
NDVI_T1	FarmCPU	BS00067828_51	2B	754661130	0.00089225	3.05
NDVI_T1	GLM	BS00102480_51	2B	157694228	0.00055941	3.26
NDVI_T2	FarmCPU	Tdurum_contig20589_247	2B	238961085	4.48E-05	4.35
NDVI_T2	FarmCPU	RAC875_c63112_460	2B	239646009	4.48E-05	4.35
NDVI_T2	FarmCPU	BS00072379_51	2B	249198797	0.00034753	3.46
NDVI_T2	FarmCPU	Ku_c9369_1965	2B	695374866	0.0004925	3.31
NDVI_T2	FarmCPU	BS00064055_51	2B	774831009	0.00087211	3.06
NDVI_T2	FarmCPU	BS00064836_51	2B	774831016	0.00065008	3.19
NDVI_T2	GLM	Tdurum_contig20589_247	2B	238961085	6.49E-05	4.19
NDVI_T2	GLM	RAC875_c63112_460	2B	239646009	6.49E-05	4.19
NDVI_T2	GLM	BS00072379_51	2B	249198797	0.00054364	3.27
NDVI_T2	GLM	Ku_c9369_1965	2B	695374866	0.00060249	3.23
NDVI_T2	GLM	BS00064055_51	2B	774831009	0.00054293	3.27
NDVI_T2	GLM	BS00025106_51	2B	787742888	0.00013031	3.89
NDVI_T2	GLM	RAC875_c10626_2089	2B	788655567	0.00013031	3.89
NDVI_T2	GLM	wsnp_Ex_c31064_39902843	2B	789867336	0.00016552	3.79
NDVI_T2	GLM	tplb0053o16_838	2B	790586417	0.00028795	3.55
NDVI_T2	GLM	BS00056645_51	2B	793148630	0.00071591	3.15
NDVI_T2	MLM	Tdurum_contig20589_247	2B	238961085	0.00035737	3.45
NDVI_T2	MLM	RAC875_c63112_460	2B	239646009	0.00035737	3.45

PH_C	FarmCPU	Kukri_c22216_846	2B	412665110	0.00080629	3.1
PH_C	FarmCPU	wsnp_Ex_rep_c69340_68274022	2B	423836995	0.00029379	3.54
PH_C	FarmCPU	wsnp_CAP11_c1820_985143	2B	782533975	0.0001956	3.71
PH_C	GLM	Ex_c67257_2556	2B	139816493	0.00083235	3.08
PH_C	GLM	wsnp_CAP11_c1820_985143	2B	782533975	0.00049924	3.31
PH_T1	FarmCPU	BS00009604_51	2B	47172739	0.0001642	3.79
PH_T1	FarmCPU	RAC875_c95948_614	2B	69370617	0.00049024	3.31
PH_T1	FarmCPU	RAC875_c22619_364	2B	72577204	0.0002418	3.62
PH_T1	FarmCPU	wsnp_Ex_c6099_10674406	2B	72578208	0.0002418	3.62
PH_T1	FarmCPU	wsnp_Ra_c8489_14382125	2B	72578758	0.0002418	3.62
PH_T1	FarmCPU	Kukri_rep_c101093_572	2B	72580177	0.00049024	3.31
PH_T1	FarmCPU	Kukri_c29272_363	2B	75693531	0.00074368	3.13
PH_T1	FarmCPU	RAC875_c8780_441	2B	76929509	0.00074368	3.13
PH_T1	FarmCPU	Excalibur_c7136_823	2B	77783224	0.00074368	3.13
PH_T1	GLM	BS00009604_51	2B	47172739	0.00021427	3.67
PH_T1	GLM	RAC875_c95948_614	2B	69370617	0.00045367	3.35
PH_T1	GLM	RAC875_c22619_364	2B	72577204	0.00020112	3.7
PH_T1	GLM	wsnp_Ex_c6099_10674406	2B	72578208	0.00020112	3.7
PH_T1	GLM	wsnp_Ra_c8489_14382125	2B	72578758	0.00020112	3.7
PH_T1	GLM	Kukri_rep_c101093_572	2B	72580177	0.00045367	3.35
PH_T1	GLM	Kukri_c29272_363	2B	75693531	0.00052626	3.28
PH_T1	GLM	RAC875_c8780_441	2B	76929509	0.00052626	3.28
PH_T1	GLM	Excalibur_c7136_823	2B	77783224	0.00052626	3.28
PH_T1	GLM	CAP7_c12727_215	2B	706727024	0.00052992	3.28
PH_T1	MLM	BS00009604_51	2B	47172739	0.0005124	3.3
PH_T1	MLM	RAC875_c22619_364	2B	72577204	0.00067841	3.17
PH_T1	MLM	wsnp_Ex_c6099_10674406	2B	72578208	0.00067841	3.17
PH_T1	MLM	wsnp_Ra_c8489_14382125	2B	72578758	0.00067841	3.17
PH_T2	FarmCPU	Kukri_rep_c101462_172	2B	159891911	0.00017111	3.77
PH_T2	FarmCPU	Tdurum_contig55699_246	2B	683028910	0.00099248	3.01
PH_T2	FarmCPU	Excalibur_c47745_63	2B	704721642	9.07E-05	4.05
PH_T2	FarmCPU	CAP7_c12727_215	2B	706727024	0.0005139	3.29
PH_T2	FarmCPU	wsnp_CAP7_c317_172502	2B	731000103	0.00017917	3.75
PH_T2	FarmCPU	Tdurum_contig93103_284	2B	737689251	0.00028257	3.55
PH_T2	GLM	Kukri_rep_c101462_172	2B	159891911	0.0002268	3.65
PH_T2	GLM	Tdurum_contig55699_246	2B	683028910	0.00083479	3.08
PH_T2	GLM	Excalibur_c47745_63	2B	704721642	0.0001102	3.96
PH_T2	GLM	CAP7_c12727_215	2B	706727024	0.00021111	3.68
PH_T2	GLM	wsnp_CAP7_c317_172502	2B	731000103	0.00026534	3.58
PH_T2	GLM	Tdurum_contig93103_284	2B	737689251	0.00017652	3.76
PH_T2	GLM	Tdurum_contig47_185	2B	738338776	0.00068664	3.17
PH_T2	MLM	Kukri_rep_c101462_172	2B	159891911	0.00061337	3.22
PH_T2	MLM	Excalibur_c47745_63	2B	704721642	0.0003767	3.43
PH_T2	MLM	wsnp_CAP7_c317_172502	2B	731000103	0.00065389	3.19
PH_T2	MLM	Tdurum_contig93103_284	2B	737689251	0.00091303	3.04
T.P_C	FarmCPU	wsnp_Ra_rep_c117300_96881829	2B	28367745	0.00084606	3.08

T.P_C	FarmCPU	Tdurum_contig30210_226	2B	28415893	2.19E-05	4.66
T.P_C	FarmCPU	Tdurum_contig12589_325	2B	516531745	0.00016055	3.8
T.P_C	GLM	wsnp_Ra_rep_c117300_96881829	2B	28367745	0.0008405	3.08
T.P_C	GLM	Tdurum_contig30210_226	2B	28415893	3.97E-05	4.41
T.P_C	GLM	Tdurum_contig12589_325	2B	516531745	0.00019915	3.71
T.P_C	MLM	Tdurum_contig30210_226	2B	28415893	0.00012806	3.9
T.P_C	MLM	Tdurum_contig12589_325	2B	516531745	0.00050421	3.3
T.p_T2	FarmCPU	Tdurum_contig30210_226	2B	28415893	0.00041513	3.39
T.p_T2	FarmCPU	Excalibur_rep_c88533_231	2B	797243338	0.00080155	3.1
T.p_T2	GLM	Tdurum_contig30210_226	2B	28415893	0.00057642	3.24
T.p_T2	GLM	RAC875_c19042_2102	2B	796803386	0.00053698	3.28
HI_C	FarmCPU	Tdurum_contig10380_87	2B	651358172	0.00089283	3.05
HI_C	FarmCPU	Tdurum_contig84620_175	2B	653620484	0.00061043	3.22
HI_T1	GLM	GENE-0872_343	2B	604999207	0.00066929	3.18
HI_T1	GLM	GENE-0777_105	2B	760890875	0.00077473	3.12
BM_C	FarmCPU	Excalibur_rep_c67599_242	2D	650327186	0.00021885	3.66
BM_C	GLM	Excalibur_rep_c67599_242	2D	650327186	0.00021796	3.67
BM_C	MLM	Excalibur_rep_c67599_242	2D	650327186	0.00070166	3.16
FLA_T1	GLM	RAC875_rep_c73201_205	2D	79986387	0.00093201	3.04
FLA_T1	GLM	BS00021865_51	2D	81651764	0.00089992	3.05
FLA_T2	FarmCPU	BobWhite_c38001_579	2D	589496497	0.00079724	3.1
GpS_C	FarmCPU	Kukri_c13708_204	2D	14401084	0.00024096	3.62
GpS_C	FarmCPU	wsnp_Ex_rep_c67011_65463819	2D	14401234	0.00024096	3.62
GpS_C	FarmCPU	Kukri_c11809_824	2D	14896719	0.00033724	3.48
GpS_C	GLM	Kukri_c13708_204	2D	14401084	0.00035152	3.46
GpS_C	GLM	wsnp_Ex_rep_c67011_65463819	2D	14401234	0.00035152	3.46
GpS_C	GLM	Kukri_c11809_824	2D	14896719	0.0004894	3.32
GpS_C	MLM	Kukri_c13708_204	2D	14401084	0.00067659	3.17
GpS_C	MLM	wsnp_Ex_rep_c67011_65463819	2D	14401234	0.00067659	3.17
GpS_C	MLM	Kukri_c11809_824	2D	14896719	0.00086658	3.07
GY_C	FarmCPU	Excalibur_rep_c67599_242	2D	650327186	0.00049747	3.31
GY_C	GLM	Excalibur_rep_c67599_242	2D	650327186	0.00019848	3.71
GY_T1	FarmCPU	IACX11138	2D	3756210	0.00056803	3.25
GY_T1	GLM	IACX11138	2D	3756210	0.00097637	3.02
GY_T2	GLM	wsnp_CAP12_c455_248396	2D	14778680	0.00097571	3.02
PH_C	FarmCPU	Ex_c2115_3369	2D	435045122	0.00011948	3.93
PH_C	FarmCPU	BS00036456_51	2D	592788886	1.87E-05	4.73
PH_C	GLM	Ex_c2115_3369	2D	435045122	0.00056108	3.26
PH_C	GLM	BS00036456_51	2D	592788886	0.0001138	3.95
PH_C	MLM	Ex_c2115_3369	2D	435045122	0.00092649	3.04
PH_C	MLM	BS00036456_51	2D	592788886	0.00023176	3.64
PH_T1	FarmCPU	Kukri_c59585_560	2D	45535709	0.00062983	3.21
PH_T1	FarmCPU	Kukri_c27574_725	2D	48033359	0.00074368	3.13
PH_T1	GLM	Kukri_c59585_560	2D	45535709	0.00051896	3.29
PH_T1	GLM	Kukri_c27574_725	2D	48033359	0.00052626	3.28
PH_T2	FarmCPU	BS00036456_51	2D	592788886	0.0001942	3.72

PH_T2	GLM	BobWhite_c10627_354	2D	569949142	0.00082273	3.09
PH_T2	GLM	BS00036456_51	2D	592788886	0.00019368	3.72
PH_T2	MLM	BS00036456_51	2D	592788886	0.00050744	3.3
T.P_C	FarmCPU	wsnp_Ex_c12250_19568265	2D	13909771	0.00075248	3.13
HI_T1	GLM	Ex_c10068_1509	2D	619416257	0.00077473	3.12
BM_C	FarmCPU	Kukri_c12212_182	3A	729575982	0.00023546	3.63
BM_C	FarmCPU	wsnp_RFL_Contig3344_3442711	3A	36230890	0.00087559	3.06
BM_C	FarmCPU	RAC875_c99055_69	3A	728322677	0.00094337	3.03
BM_C	FarmCPU	Tdurum_contig31379_183	3A	36228890	0.00098626	3.01
BM_C	GLM	Tdurum_contig31379_183	3A	36228890	0.00059927	3.23
BM_C	GLM	wsnp_RFL_Contig3344_3442711	3A	36230890	0.00048056	3.32
BM_C	GLM	RAC875_c75448_80	3A	600928588	0.00076319	3.12
BM_C	GLM	Kukri_c12212_182	3A	729575982	0.00016296	3.79
BM_C	MLM	Kukri_c12212_182	3A	729575982	0.00075638	3.13
BM_T1	FarmCPU	BS00047668_51	3A	639148445	0.00033884	3.48
BM_T1	GLM	BS00047668_51	3A	639148445	0.00035724	3.45
BM_T1	MLM	BS00047668_51	3A	639148445	0.00086973	3.07
CHL_C	FarmCPU	Kukri_c51666_401	3A	739520589	0.00091745	3.04
CHL_C	GLM	BS00093889_51	3A	724319940	0.00046513	3.34
CHL_T1	GLM	RAC875_c15970_89	3A	701238651	0.00088338	3.06
FLA_T2	GLM	BobWhite_rep_c61884_158	3A	513605063	0.00097653	3.02
GpS_T1	FarmCPU	Tdurum_contig12371_248	3A	47825498	0.0009163	3.04
GpS_T1	GLM	Tdurum_contig12371_248	3A	47825498	0.00099771	3.01
GY_T2	FarmCPU	Kukri_c49280_230	3A	20134735	3.51E-06	5.46
GY_T2	GLM	Kukri_rep_c89183_256	3A	8685971	0.00038338	3.42
GY_T2	GLM	wsnp_Ex_rep_c67702_66370241	3A	8865520	0.00064673	3.19
GY_T2	GLM	Ra_c73278_1234	3A	10301113	0.00010361	3.99
GY_T2	GLM	Kukri_rep_c75764_60	3A	20134479	0.00016991	3.77
GY_T2	GLM	Kukri_c49280_230	3A	20134735	8.09E-05	4.1
GY_T2	MLM	Ra_c73278_1234	3A	10301113	0.00055987	3.26
GY_T2	MLM	Kukri_c49280_230	3A	20134735	0.00055088	3.26
PH_C	FarmCPU	BS00098840_51	3A	140043235	0.00044015	3.36
PH_C	FarmCPU	IAAV8990	3A	375817168	0.00074351	3.13
PH_C	FarmCPU	Ra_c5515_2396	3A	514111849	6.47E-05	4.19
PH_C	GLM	BS00098840_51	3A	140043235	0.00089072	3.06
PH_C	GLM	IAAV8990	3A	375817168	0.0007993	3.1
PH_C	GLM	Ra_c5515_2396	3A	514111849	0.00013367	3.88
PH_C	MLM	Ra_c5515_2396	3A	514111849	0.00061121	3.22
PH_T2	FarmCPU	Excalibur_c11079_749	3A	32201535	2.27E-06	5.65
PH_T2	FarmCPU	Ra_c38505_544	3A	558890611	0.00056607	3.25
PH_T2	FarmCPU	BobWhite_c21423_295	3A	738131396	0.00017111	3.77
PH_T2	GLM	Excalibur_c11079_749	3A	32201535	3.73E-06	5.43
PH_T2	GLM	Ra_c38505_544	3A	558890611	0.00033418	3.48
PH_T2	GLM	BobWhite_c21423_295	3A	738131396	0.0002268	3.65
PH_T2	MLM	Excalibur_c11079_749	3A	32201535	3.35E-05	4.48
PH_T2	MLM	BobWhite_c21423_295	3A	738131396	0.00061337	3.22

T.p_T2	FarmCPU	IAAV902	3A	574256873	0.00050007	3.31
HI_C	FarmCPU	Tdurum_contig99640_243	3A	40363548	0.00059629	3.23
HI_C	FarmCPU	tplb0036i05_182	3A	714951111	0.00017104	3.77
HI_C	GLM	Tdurum_contig99640_243	3A	40363548	0.00094299	3.03
HI_C	GLM	tplb0036i05_182	3A	714951111	0.00033935	3.47
HI_C	MLM	tplb0036i05_182	3A	714951111	0.00052783	3.28
HI_T1	GLM	BS00092728_51	3A	1308960	0.00021634	3.67
HI_T1	GLM	wsnp_Ra_c16846_25598885	3A	135989086	0.00085162	3.07
HI_T1	GLM	Kukri_c51247_322	3A	140043493	0.00085162	3.07
HI_T1	GLM	BS00110350_51	3A	697456650	0.00077473	3.12
HI_T1	GLM	Ku_c6126_1140	3A	700591347	0.00030505	3.52
HI_T1	GLM	BobWhite_c5924_503	3A	729949749	0.00066929	3.18
HI_T1	GLM	Tdurum_contig5009_735	3A	741240361	5.83E-05	4.24
HI_T1	MLM	BS00092728_51	3A	1308960	0.00026766	3.58
HI_T1	MLM	Tdurum_contig5009_735	3A	741240361	8.62E-05	4.07
NAE_T1	FarmCPU	RAC875_c29241_165	3A	44629357	0.00072623	3.14
NAE_T1	FarmCPU	wsnp_Ex_c15264_23484775	3A	44634185	0.00044152	3.36
NAE_T1	GLM	wsnp_Ex_c15264_23484775	3A	44634185	0.00084189	3.08
FLA_C	FarmCPU	BS00065429_51	3B	71144369	0.00030557	3.52
FLA_C	FarmCPU	Jagger_c2876_255	3B	251651252	0.00054479	3.27
FLA_C	GLM	Ku_c14750_566	3B	51949890	0.00025684	3.6
FLA_T1	FarmCPU	Jagger_c2876_255	3B	251651252	0.0008004	3.1
FLA_T1	GLM	Jagger_c2876_255	3B	251651252	0.000721	3.15
FLA_T1	GLM	Kukri_c21759_1035	3B	382003089	0.00070429	3.16
FLA_T2	FarmCPU	wsnp_Ex_c8360_14085858	3B	5953163	0.0005385	3.27
FLA_T2	FarmCPU	BS00095247_51	3B	197246108	0.00084739	3.08
FLA_T2	GLM	wsnp_Ex_c8360_14085858	3B	5953163	0.00027246	3.57
FLA_T2	GLM	BS00095247_51	3B	197246108	0.00078485	3.11
GpS_C	GLM	Kukri_c17467_2711	3B	761576738	0.0003097	3.51
GY_T1	FarmCPU	RFL_Contig3626_521	3B	1617465	9.27E-05	4.04
GY_T1	GLM	RFL_Contig3626_521	3B	1617465	9.33E-05	4.04
GY_T1	MLM	RFL_Contig3626_521	3B	1617465	0.00034128	3.47
GY_T2	FarmCPU	RAC875_c32005_247	3B	34303152	0.00052098	3.29
NDVI_C	FarmCPU	RAC875_c25375_236	3B	132904629	0.00061035	3.22
NDVI_T1	FarmCPU	Kukri_c60633_121	3B	756095398	0.00092041	3.04
NDVI_T1	GLM	Kukri_c60633_121	3B	756095398	0.00064426	3.2
NDVI_T2	FarmCPU	BS00095515_51	3B	772397461	3.14E-05	4.51
NDVI_T2	FarmCPU	Kukri_rep_c68685_795	3B	784488285	0.00021737	3.67
NDVI_T2	GLM	BS00095515_51	3B	772397461	4.17E-05	4.38
NDVI_T2	GLM	Kukri_rep_c68685_795	3B	784488285	0.00027356	3.57
NDVI_T2	MLM	BS00095515_51	3B	772397461	0.00019913	3.71
NDVI_T2	MLM	Kukri_rep_c68685_795	3B	784488285	0.00061772	3.21
PH_C	FarmCPU	wsnp_Ra_rep_c70261_68008978	3B	730233447	0.00071441	3.15
PH_C	GLM	wsnp_Ra_rep_c70261_68008978	3B	730233447	0.00074668	3.13
PH_T1	FarmCPU	wsnp_Ra_rep_c70261_68008978	3B	730233447	0.00068976	3.17
PH_T1	GLM	Tdurum_contig11192_373	3B	5674447	0.00040004	3.4

PH_T1	GLM	wsnp_Ku_c12698_20441325	3B	5674822	0.00064806	3.19
PH_T1	GLM	CAP7_c9234_109	3B	5952324	0.00068181	3.17
PH_T1	GLM	wsnp_Ra_rep_c70261_68008978	3B	730233447	0.00071325	3.15
HI_T1	FarmCPU	JD_c23336_253	3B	9170025	8.48E-06	5.08
HI_T1	GLM	JD_c23336_253	3B	9170025	1.28E-06	5.9
HI_T1	GLM	RAC875_rep_c70009_157	3B	128669285	0.00035047	3.46
HI_T1	GLM	wsnp_RFL_Contig2011_1216801	3B	473183926	0.00051845	3.29
HI_T1	GLM	Kukri_rep_c83522_342	3B	820286771	1.24E-05	4.91
HI_T1	MLM	JD_c23336_253	3B	9170025	7.99E-06	5.1
HI_T1	MLM	Kukri_rep_c83522_342	3B	820286771	0.0001333	3.88
FLA_C	GLM	Kukri_c73725_218	3D	31846889	0.00025684	3.6
GY_T2	GLM	RAC875_c29099_540	3D	2627630	0.00062504	3.21
PH_T1	FarmCPU	Excalibur_c25515_95	3D	28331150	0.00011974	3.93
PH_T1	GLM	Excalibur_c25515_95	3D	28331150	3.47E-05	4.46
PH_T1	MLM	Excalibur_c25515_95	3D	28331150	0.00040886	3.39
PH_T2	FarmCPU	BobWhite_c621_1218	3D	32204706	2.27E-06	5.65
PH_T2	GLM	BobWhite_c621_1218	3D	32204706	3.73E-06	5.43
PH_T2	MLM	BobWhite_c621_1218	3D	32204706	3.35E-05	4.48
T.P_C	FarmCPU	Ra_c23432_639	3D	559184550	6.31E-05	4.2
T.P_C	GLM	Ra_c23432_639	3D	559184550	9.81E-05	4.01
T.P_C	MLM	Ra_c23432_639	3D	559184550	0.00026131	3.59
HI_C	FarmCPU	IAAV4876	3D	2627250	0.00096343	3.02
HI_T1	FarmCPU	wsnp_Ex_rep_c66380_64574083	3D	606883054	1.44E-05	4.85
HI_T1	GLM	Kukri_c4230_398	3D	606862789	1.24E-05	4.91
HI_T1	GLM	Ra_c6639_1170	3D	606880474	1.24E-05	4.91
HI_T1	GLM	wsnp_Ex_rep_c66380_64574083	3D	606883054	1.24E-05	4.91
HI_T1	GLM	JD_c42309_341	3D	607001306	1.24E-05	4.91
HI_T1	MLM	Kukri_c4230_398	3D	606862789	0.0001333	3.88
HI_T1	MLM	Ra_c6639_1170	3D	606880474	0.0001333	3.88
HI_T1	MLM	wsnp_Ex_rep_c66380_64574083	3D	606883054	0.0001333	3.88
HI_T1	MLM	JD_c42309_341	3D	607001306	0.0001333	3.88
CHL_C	FarmCPU	Ra_c60252_914	4A	708568095	0.00096634	3.02
GpS_C	FarmCPU	wsnp_Ku_rep_c102728_89637829	4A	65461775	0.00017646	3.76
GpS_C	GLM	wsnp_Ku_rep_c102728_89637829	4A	65461775	0.00022895	3.65
GpS_C	MLM	wsnp_Ku_rep_c102728_89637829	4A	65461775	0.00053966	3.27
GY_C	FarmCPU	GENE-2825_442	4A	6028711	0.00072698	3.14
GY_T2	GLM	RAC875_c7978_362	4A	48620433	1.93E-06	5.72
GY_T2	GLM	BS00021957_51	4A	693278257	0.00097049	3.02
GY_T2	MLM	RAC875_c7978_362	4A	48620433	9.58E-06	5.02
GY_T2	MLM	BS00021957_51	4A	693278257	0.00083786	3.08
NDVI_T2	FarmCPU	Ra_c662_521	4A	735503358	0.00070862	3.15
NDVI_T2	GLM	Excalibur_c9370_966	4A	632858990	0.00068899	3.17
NDVI_T2	GLM	Ra_c662_521	4A	735503358	0.00072549	3.14
PH_C	FarmCPU	RAC875_c21369_425	4A	606365856	0.00091792	3.04
PH_C	FarmCPU	wsnp_Ku_c4342_7887834	4A	606366006	0.00082928	3.09
PH_C	FarmCPU	Kukri_c19883_365	4A	732518611	0.00032651	3.49

PH_C	GLM	RAC875_c21369_425	4A	606365856	0.00065252	3.19
PH_C	GLM	wsnp_Ku_c4342_7887834	4A	606366006	0.0006724	3.18
PH_C	GLM	Kukri_c19883_365	4A	732518611	0.00030881	3.52
PH_T2	FarmCPU	BobWhite_c20382_117	4A	738750567	0.00057896	3.24
PH_T2	GLM	Tdurum_contig75819_1309	4A	712864877	0.00055156	3.26
PH_T2	GLM	RFL_Contig3841_2595	4A	712864977	0.00060144	3.23
PH_T2	GLM	Tdurum_contig75819_1471	4A	712865180	0.00029822	3.53
PH_T2	GLM	RFL_Contig3841_2433	4A	712865280	0.00060144	3.23
PH_T2	GLM	BS00111039_51	4A	717964838	0.00095788	3.02
PH_T2	GLM	BobWhite_c20382_117	4A	738750567	0.00028431	3.55
T.P_C	FarmCPU	BS00068244_51	4A	46125611	0.00090578	3.05
T.P_C	FarmCPU	RAC875_c9318_401	4A	46128425	0.00076629	3.12
T.P_C	GLM	RAC875_c9318_401	4A	46128425	0.00093709	3.03
T.P_C	GLM	Tdurum_contig75819_1309	4A	712864877	0.00059741	3.23
T.P_C	GLM	RFL_Contig3841_2595	4A	712864977	0.00031587	3.51
T.P_C	GLM	RFL_Contig3841_2433	4A	712865280	0.00031587	3.51
T.P_C	GLM	BS00075048_51	4A	713519432	0.00078324	3.11
T.P_C	GLM	BS00075049_51	4A	713519474	0.00078324	3.11
T.P_C	GLM	BS00045554_51	4A	713523345	9.65E-05	4.02
T.P_C	GLM	BS00045555_51	4A	713523348	4.06E-05	4.4
T.p_T1	GLM	BS00045554_51	4A	713523345	0.00045623	3.35
T.p_T1	GLM	BS00045555_51	4A	713523348	0.00024718	3.61
T.p_T1	GLM	Ex_c14135_1819	4A	714102110	0.00048777	3.32
T.p_T2	FarmCPU	BS00009970_51	4A	45338226	0.00077575	3.12
T.p_T2	FarmCPU	wsnp_JD_c5499_6647799	4A	45338373	0.00077575	3.12
T.p_T2	FarmCPU	Kukri_rep_c75204_1421	4A	313638809	0.00077937	3.11
T.p_T2	FarmCPU	IACX1427	4A	381220545	0.00077937	3.11
T.p_T2	GLM	BS00009970_51	4A	45338226	0.00089732	3.05
T.p_T2	GLM	wsnp_JD_c5499_6647799	4A	45338373	0.00089732	3.05
T.p_T2	GLM	BS00045554_51	4A	713523345	0.00096723	3.02
T.p_T2	GLM	BS00045555_51	4A	713523348	0.00027151	3.57
T.p_T2	GLM	Ex_c14135_1819	4A	714102110	0.00066987	3.18
T.p_T2	GLM	BS00111039_51	4A	717964838	0.0004418	3.36
HI_T1	GLM	RAC875_c59673_188	4A	681669073	7.96E-06	5.1
HI_T1	GLM	RAC875_c59673_500	4A	681670845	7.96E-06	5.1
HI_T1	GLM	Excalibur_c4325_1150	4A	684616549	0.00010906	3.97
HI_T1	MLM	RAC875_c59673_188	4A	681669073	0.00010662	3.98
HI_T1	MLM	RAC875_c59673_500	4A	681670845	0.00010662	3.98
HI_T1	MLM	Excalibur_c4325_1150	4A	684616549	0.00043699	3.36
NAE_T2	FarmCPU	Ku_c766_1798	4A	27674198	0.0007486	3.13
NAE_T2	GLM	Ku_c766_1798	4A	27674198	0.00072749	3.14
BM_C	FarmCPU	wsnp_Ex_rep_c67159_65649966	4B	637390195	0.00015819	3.81
BM_C	FarmCPU	BS00064032_51	4B	144927412	0.0005556	3.26
BM_C	GLM	BS00064032_51	4B	144927412	0.00060577	3.22
BM_C	GLM	wsnp_Ex_rep_c67159_65649966	4B	637390195	0.00011244	3.95
BM_C	MLM	wsnp_Ex_rep_c67159_65649966	4B	637390195	0.00054653	3.27

BM_T2	FarmCPU	BS00011510_51	4B	16056666	0.00055495	3.26
BM_T2	FarmCPU	Ku_c63300_1309	4B	21556672	0.00068533	3.17
BM_T2	FarmCPU	Kukri_c6242_147	4B	22902515	0.00063173	3.2
BM_T2	GLM	BS00011510_51	4B	16056666	0.00072812	3.14
BM_T2	GLM	Ku_c63300_1309	4B	21556672	0.00082102	3.09
BM_T2	GLM	Kukri_c6242_147	4B	22902515	0.00069232	3.16
FLA_C	GLM	Kukri_c31350_287	4B	301871347	0.00020969	3.68
FLA_T1	FarmCPU	Excalibur_c57766_92	4B	165019046	0.00081078	3.1
FLA_T1	GLM	Excalibur_c57766_92	4B	165019046	0.0004798	3.32
GpS_C	FarmCPU	IACX5783	4B	60203635	0.00052085	3.29
GpS_C	FarmCPU	Tdurum_contig1664_212	4B	60637559	0.00019541	3.71
GpS_C	GLM	IACX5783	4B	60203635	0.00054258	3.27
GpS_C	GLM	Tdurum_contig1664_212	4B	60637559	0.00021961	3.66
GpS_C	MLM	Tdurum_contig1664_212	4B	60637559	0.00058096	3.24
GY_C	FarmCPU	Kukri_rep_c78644_408	4B	650634817	0.00022276	3.66
GY_C	GLM	Kukri_rep_c78644_408	4B	650634817	0.00030373	3.52
GY_C	GLM	Excalibur_c1273_142	4B	657641927	0.00043136	3.37
GY_T2	FarmCPU	CAP11_rep_c4893_84	4B	10437558	0.00024467	3.62
GY_T2	GLM	Tdurum_contig30760_393	4B	3861016	0.00069804	3.16
GY_T2	GLM	CAP11_rep_c4893_84	4B	10437558	4.56E-05	4.35
GY_T2	GLM	BS00022646_51	4B	613317766	0.00012776	3.9
GY_T2	MLM	CAP11_rep_c4893_84	4B	10437558	6.18E-05	4.21
GY_T2	MLM	BS00022646_51	4B	613317766	0.0006666	3.18
NAE_T2	FarmCPU	Excalibur_c14401_404	4B	6149014	0.00059658	3.23
NAE_T2	FarmCPU	BS00062304_51	4B	660719556	0.00056361	3.25
NAE_T2	GLM	Excalibur_c14401_404	4B	6149014	0.00059402	3.23
NAE_T2	GLM	BS00062304_51	4B	660719556	0.00045755	3.34
GpS_C	FarmCPU	Excalibur_c29496_799	4D	475027917	0.00029844	3.53
GpS_C	GLM	Excalibur_c29496_799	4D	475027917	0.00040009	3.4
GpS_C	MLM	Excalibur_c29496_799	4D	475027917	0.00079165	3.11
GpS_T2	FarmCPU	IAAV5850	4D	110798223	0.00082993	3.09
GpS_T2	GLM	IAAV5850	4D	110798223	0.00058334	3.24
GY_T2	FarmCPU	IAAV1674	4D	1876548	1.07E-05	4.98
NDVI_T1	GLM	Ex_c6665_1067	4D	65075379	0.00073559	3.14
T.p_T2	FarmCPU	RAC875_c45385_212	4D	116273420	0.00071688	3.15
T.p_T2	FarmCPU	GENE-2463_463	4D	119747035	0.00077937	3.11
NAE_T2	FarmCPU	RAC875_c215_329	4D	1822357	0.00068289	3.17
NAE_T2	GLM	RAC875_c215_329	4D	1822357	0.0006389	3.2
BM_C	FarmCPU	wsnp_Ex_c11573_18650189	5A	482372063	0.00018695	3.73
BM_C	FarmCPU	BobWhite_rep_c63943_76	5A	547415426	0.00086319	3.07
BM_C	GLM	wsnp_Ex_c11573_18650189	5A	482372063	0.00015326	3.82
BM_C	GLM	BobWhite_rep_c63943_76	5A	547415426	0.00074431	3.13
BM_C	MLM	wsnp_Ex_c11573_18650189	5A	482372063	0.00061904	3.21
BM_T2	GLM	Excalibur_c42255_425	5A	702166658	0.00052868	3.28
BM_T2	GLM	BS00068108_51	5A	702461315	0.00052868	3.28
FLA_T1	FarmCPU	IAAV2194	5A	69846802	0.0002325	3.64

FLA_T1	GLM	IAAV2194	5A	69846802	0.00014342	3.85
FLA_T1	MLM	IAAV2194	5A	69846802	0.00065929	3.19
FLA_T2	FarmCPU	Tdurum_contig12204_1131	5A	705442972	0.00022153	3.66
FLA_T2	GLM	Tdurum_contig12204_1131	5A	705442972	6.90E-05	4.17
FLA_T2	MLM	Tdurum_contig12204_1131	5A	705442972	0.00063626	3.2
GpS_C	FarmCPU	JD_c5000_410	5A	526619421	0.00082358	3.09
GpS_C	GLM	JD_c5000_410	5A	526619421	0.0009924	3.01
GY_C	FarmCPU	Excalibur_c112658_300	5A	457521085	0.00014191	3.85
GY_C	FarmCPU	wsnp_Ex_c2474_4619730	5A	457521276	0.00080662	3.1
GY_C	FarmCPU	wsnp_Ex_c23795_33033010	5A	679666183	0.0009909	3.01
GY_C	GLM	wsnp_Ex_rep_c67292_65834396	5A	456608086	0.00058452	3.24
GY_C	GLM	BS00075308_51	5A	457089670	0.00052777	3.28
GY_C	GLM	Excalibur_c112658_300	5A	457521085	6.19E-05	4.21
GY_C	GLM	wsnp_Ex_c2474_4619730	5A	457521276	0.00078484	3.11
GY_C	MLM	Excalibur_c112658_300	5A	457521085	0.00028656	3.55
GY_T1	FarmCPU	Excalibur_c112658_300	5A	457521085	0.00057508	3.25
GY_T1	GLM	Excalibur_c112658_300	5A	457521085	0.00037356	3.43
GY_T2	FarmCPU	IAAV4830	5A	457437330	0.00063176	3.2
GY_T2	FarmCPU	wsnp_Ex_c472_935980	5A	568269292	3.66E-07	6.44
GY_T2	GLM	wsnp_Ku_c9559_15999945	5A	8243240	0.00029392	3.54
GY_T2	GLM	Excalibur_c36501_188	5A	9325617	0.00029392	3.54
GY_T2	MLM	wsnp_Ku_c9559_15999945	5A	8243240	0.00044199	3.36
GY_T2	MLM	Excalibur_c36501_188	5A	9325617	0.00044199	3.36
NDVI_T1	FarmCPU	BS00023008_51	5A	8059358	0.0009928	3.01
NDVI_T1	FarmCPU	BobWhite_c5540_416	5A	519905749	0.00024071	3.62
NDVI_T1	GLM	BS00023008_51	5A	8059358	0.0005445	3.27
NDVI_T1	GLM	Kukri_c23694_370	5A	17222966	0.00040796	3.39
NDVI_T1	GLM	BobWhite_c5540_416	5A	519905749	0.00022376	3.66
NDVI_T1	MLM	BobWhite_c5540_416	5A	519905749	0.00065842	3.19
PH_T2	FarmCPU	Kukri_rep_c77867_217	5A	47662050	0.00017111	3.77
PH_T2	GLM	Kukri_rep_c77867_217	5A	47662050	0.0002268	3.65
PH_T2	MLM	Kukri_rep_c77867_217	5A	47662050	0.00061337	3.22
T.p_T1	FarmCPU	Kukri_c2621_610	5A	467379070	0.00028454	3.55
T.p_T1	FarmCPU	Kukri_c17430_972	5A	468467263	0.00028454	3.55
T.p_T1	FarmCPU	wsnp_Ex_c19647_28632894	5A	470033346	9.95E-05	4.01
T.p_T1	FarmCPU	BS00022191_51	5A	476402782	0.00015653	3.81
T.p_T1	FarmCPU	RAC875_c1219_1258	5A	476603826	0.00016412	3.79
T.p_T1	GLM	Kukri_c2621_610	5A	467379070	0.00028256	3.55
T.p_T1	GLM	Kukri_c17430_972	5A	468467263	0.00028256	3.55
T.p_T1	GLM	wsnp_Ex_c19647_28632894	5A	470033346	0.00017631	3.76
T.p_T1	GLM	BS00022191_51	5A	476402782	0.00015945	3.8
T.p_T1	GLM	RAC875_c1219_1258	5A	476603826	0.0001173	3.94
T.p_T1	MLM	Kukri_c2621_610	5A	467379070	0.00076448	3.12
T.p_T1	MLM	Kukri_c17430_972	5A	468467263	0.00076448	3.12
T.p_T1	MLM	wsnp_Ex_c19647_28632894	5A	470033346	0.00035898	3.45
T.p_T1	MLM	BS00022191_51	5A	476402782	0.00049514	3.31

T.p_T1	MLM	RAC875_c1219_1258	5A	476603826	0.0005122	3.3
T.p_T2	FarmCPU	BS00022191_51	5A	476402782	0.00011351	3.95
T.p_T2	FarmCPU	RAC875_c1219_1258	5A	476603826	0.00029731	3.53
T.p_T2	FarmCPU	BS00069175_51	5A	485598177	0.00089229	3.05
T.p_T2	GLM	BS00022191_51	5A	476402782	4.73E-05	4.33
T.p_T2	GLM	RAC875_c1219_1258	5A	476603826	7.91E-05	4.11
T.p_T2	GLM	BS00069175_51	5A	485598177	0.00086253	3.07
T.p_T2	MLM	BS00022191_51	5A	476402782	0.00031677	3.5
T.p_T2	MLM	RAC875_c1219_1258	5A	476603826	0.0005773	3.24
HI_C	GLM	BS00065693_51	5A	442352976	0.00043636	3.37
HI_T1	FarmCPU	wsnp_Ex_c905_1749059	5A	335579	0.00048141	3.32
HI_T1	FarmCPU	BobWhite_c6759_365	5A	488262509	2.33E-11	10.64
HI_T1	FarmCPU	Kukri_c60913_155	5A	568268732	8.60E-05	4.07
HI_T1	GLM	BS00040623_51	5A	391548987	4.07E-05	4.4
HI_T1	GLM	Ku_c47168_563	5A	487844932	0.00034906	3.46
HI_T1	GLM	BobWhite_c6759_365	5A	488262509	1.69E-05	4.78
HI_T1	GLM	wsnp_Ex_c7168_12311649	5A	488262635	9.30E-05	4.04
HI_T1	GLM	IAAV6488	5A	488893224	0.00055247	3.26
HI_T1	GLM	BS00065714_51	5A	691027828	0.00072756	3.14
HI_T1	MLM	BS00040623_51	5A	391548987	0.00041844	3.38
HI_T1	MLM	Ku_c47168_563	5A	487844932	0.00066363	3.18
HI_T1	MLM	BobWhite_c6759_365	5A	488262509	7.16E-05	4.15
HI_T1	MLM	wsnp_Ex_c7168_12311649	5A	488262635	0.00027639	3.56
HI_T1	MLM	IAAV6488	5A	488893224	0.000927	3.04
HI_T2	FarmCPU	Excalibur_c104037_107	5A	670820035	0.00091465	3.04
BM_T1	FarmCPU	Ra_c19198_137	5B	26916227	0.00068377	3.17
BM_T1	GLM	Ra_c19198_137	5B	26916227	0.00047218	3.33
BM_T2	GLM	IACX17304	5B	527609009	0.00079127	3.11
BM_T2	GLM	IAAV659	5B	527609162	0.00079127	3.11
CHL_C	FarmCPU	Excalibur_c55348_283	5B	439654590	0.00055733	3.26
CHL_T2	FarmCPU	BS00022773_51	5B	27821080	0.00084869	3.08
FLA_C	FarmCPU	BS00049997_51	5B	626069850	0.00023785	3.63
FLA_C	GLM	BS00049997_51	5B	626069850	0.00074834	3.13
FLA_T1	FarmCPU	GENE-0782_747	5B	56565862	6.92E-05	4.16
FLA_T1	FarmCPU	wsnp_Ex_c2904_5355509	5B	60794454	5.94E-05	4.23
FLA_T1	FarmCPU	BS00074315_51	5B	61381215	5.90E-05	4.23
FLA_T1	FarmCPU	JD_c16284_736	5B	63362199	5.94E-05	4.23
FLA_T1	FarmCPU	RAC875_c2440_755	5B	64732501	0.00019164	3.72
FLA_T1	FarmCPU	BobWhite_c10954_467	5B	64732586	0.00091588	3.04
FLA_T1	FarmCPU	BS00050709_51	5B	64733341	0.00024604	3.61
FLA_T1	FarmCPU	Ex_c1846_1818	5B	64736505	0.0002548	3.6
FLA_T1	FarmCPU	Ku_c439_1308	5B	64736528	0.0002548	3.6
FLA_T1	FarmCPU	Kukri_c439_857	5B	64736979	0.00019164	3.72
FLA_T1	FarmCPU	IAAV4252	5B	65243708	0.00019164	3.72
FLA_T1	FarmCPU	Excalibur_c5540_1197	5B	68359590	0.00019164	3.72
FLA_T1	FarmCPU	wsnp_Ku_c14252_22506286	5B	68846430	0.0002548	3.6

FLA_T1	FarmCPU	BobWhite_c15585_87	5B	68846580	9.04E-05	4.05
FLA_T1	FarmCPU	BS00067028_51	5B	70441099	5.90E-05	4.23
FLA_T1	FarmCPU	Ku_c4349_1791	5B	74336357	0.00029642	3.53
FLA_T1	FarmCPU	CAP7_c1403_70	5B	77714754	5.90E-05	4.23
FLA_T1	FarmCPU	BobWhite_rep_c55336_265	5B	79803716	0.0002325	3.64
FLA_T1	GLM	GENE-0782_747	5B	56565862	5.30E-05	4.28
FLA_T1	GLM	wsnp_Ex_c2904_5355509	5B	60794454	2.85E-05	4.55
FLA_T1	GLM	BS00074315_51	5B	61381215	5.35E-05	4.28
FLA_T1	GLM	JD_c16284_736	5B	63362199	2.85E-05	4.55
FLA_T1	GLM	RAC875_c2440_755	5B	64732501	0.00015054	3.83
FLA_T1	GLM	BobWhite_c10954_467	5B	64732586	0.0007535	3.13
FLA_T1	GLM	BS00050709_51	5B	64733341	0.00022205	3.66
FLA_T1	GLM	Ex_c1846_1818	5B	64736505	0.00023369	3.64
FLA_T1	GLM	Ku_c439_1308	5B	64736528	0.00023369	3.64
FLA_T1	GLM	Kukri_c439_857	5B	64736979	0.00015054	3.83
FLA_T1	GLM	IAAV4252	5B	65243708	0.00015054	3.83
FLA_T1	GLM	Excalibur_c5540_1197	5B	68359590	0.00015054	3.83
FLA_T1	GLM	wsnp_Ku_c14252_22506286	5B	68846430	0.00023369	3.64
FLA_T1	GLM	BobWhite_c15585_87	5B	68846580	6.53E-05	4.19
FLA_T1	GLM	BS00067028_51	5B	70441099	5.35E-05	4.28
FLA_T1	GLM	Ku_c4349_1791	5B	74336357	0.00027641	3.56
FLA_T1	GLM	CAP7_c1403_70	5B	77714754	5.35E-05	4.28
FLA_T1	GLM	BobWhite_rep_c55336_265	5B	79803716	0.00014342	3.85
FLA_T1	MLM	GENE-0782_747	5B	56565862	0.00027851	3.56
FLA_T1	MLM	wsnp_Ex_c2904_5355509	5B	60794454	0.00025055	3.61
FLA_T1	MLM	BS00074315_51	5B	61381215	0.00024955	3.61
FLA_T1	MLM	JD_c16284_736	5B	63362199	0.00025055	3.61
FLA_T1	MLM	RAC875_c2440_755	5B	64732501	0.00057292	3.25
FLA_T1	MLM	BS00050709_51	5B	64733341	0.00068714	3.17
FLA_T1	MLM	Ex_c1846_1818	5B	64736505	0.00070495	3.16
FLA_T1	MLM	Ku_c439_1308	5B	64736528	0.00070495	3.16
FLA_T1	MLM	Kukri_c439_857	5B	64736979	0.00057292	3.25
FLA_T1	MLM	IAAV4252	5B	65243708	0.00057292	3.25
FLA_T1	MLM	Excalibur_c5540_1197	5B	68359590	0.00057292	3.25
FLA_T1	MLM	wsnp_Ku_c14252_22506286	5B	68846430	0.00070495	3.16
FLA_T1	MLM	BobWhite_c15585_87	5B	68846580	0.00033559	3.48
FLA_T1	MLM	BS00067028_51	5B	70441099	0.00024955	3.61
FLA_T1	MLM	Ku_c4349_1791	5B	74336357	0.00078793	3.11
FLA_T1	MLM	CAP7_c1403_70	5B	77714754	0.00024955	3.61
FLA_T1	MLM	BobWhite_rep_c55336_265	5B	79803716	0.00065929	3.19
GY_T2	FarmCPU	Kukri_c20360_1090	5B	239396928	0.00042446	3.38
GY_T2	FarmCPU	IACX9238	5B	587127034	4.42E-06	5.36
GY_T2	GLM	IACX9238	5B	587127034	0.00014361	3.85
GY_T2	MLM	IACX9238	5B	587127034	0.00020569	3.69
NDVI_C	GLM	BS00067308_51	5B	690331053	0.00036401	3.44
NDVI_T1	FarmCPU	Kukri_c2346_1102	5B	536515507	0.00023206	3.64

NDVI_T1	FarmCPU	Excalibur_rep_c106165_238	5B	591144433	0.00023206	3.64
NDVI_T1	GLM	Kukri_c2346_1102	5B	536515507	0.00020162	3.7
NDVI_T1	GLM	Excalibur_rep_c106165_238	5B	591144433	0.00020162	3.7
NDVI_T1	MLM	Kukri_c2346_1102	5B	536515507	0.0006655	3.18
NDVI_T1	MLM	Excalibur_rep_c106165_238	5B	591144433	0.0006655	3.18
PH_C	FarmCPU	Tdurum_contig44115_132	5B	669896518	0.00066738	3.18
PH_C	FarmCPU	RAC875_c62400_267	5B	669896662	0.00059448	3.23
PH_C	FarmCPU	Tdurum_contig44115_561	5B	669897388	0.00066738	3.18
PH_C	GLM	Tdurum_contig44115_132	5B	669896518	0.00013809	3.86
PH_C	GLM	RAC875_c62400_267	5B	669896662	0.00014039	3.86
PH_C	GLM	Tdurum_contig44115_561	5B	669897388	0.00013809	3.86
PH_C	GLM	RAC875_c62400_639	5B	669897694	0.00033816	3.48
PH_C	GLM	RAC875_c62400_840	5B	669897891	0.00033816	3.48
PH_C	MLM	Tdurum_contig44115_132	5B	669896518	0.00061645	3.22
PH_C	MLM	RAC875_c62400_267	5B	669896662	0.00086743	3.07
PH_C	MLM	Tdurum_contig44115_561	5B	669897388	0.00061645	3.22
PH_T1	FarmCPU	wsnp_Ex_c3834_6971712	5B	536516487	0.00095336	3.03
PH_T1	GLM	wsnp_Ex_c3834_6971712	5B	536516487	0.00082865	3.09
PH_T2	FarmCPU	wsnp_Ku_c12562_20256747	5B	477667808	0.00024023	3.62
PH_T2	GLM	wsnp_Ku_c12562_20256747	5B	477667808	0.00021712	3.67
PH_T2	MLM	wsnp_Ku_c12562_20256747	5B	477667808	0.00071709	3.15
HI_C	GLM	BobWhite_rep_c65811_114	5B	484735491	0.00096463	3.02
HI_T1	GLM	BS00100707_51	5B	638508116	0.00059015	3.23
HI_T1	GLM	Tdurum_contig44115_132	5B	669896518	0.00042388	3.38
HI_T1	GLM	Tdurum_contig44115_561	5B	669897388	0.00042388	3.38
HI_T1	GLM	RAC875_c62400_639	5B	669897694	0.00063632	3.2
HI_T1	GLM	RAC875_c62400_840	5B	669897891	0.00063632	3.2
HI_T1	GLM	BS00095157_51	5B	670246207	0.00070827	3.15
HI_T1	GLM	Tdurum_contig42526_73	5B	694519049	0.00066929	3.18
HI_T1	GLM	RAC875_rep_c96433_140	5B	694519359	0.00029362	3.54
HI_T1	GLM	Excalibur_rep_c100012_1145	5B	695659830	7.06E-05	4.16
HI_T1	MLM	BS00022960_51	5B	507588197	0.00095498	3.03
HI_T1	MLM	RAC875_rep_c96433_140	5B	694519359	0.00042878	3.37
HI_T1	MLM	Excalibur_rep_c100012_1145	5B	695659830	0.0002256	3.65
HI_T2	FarmCPU	Excalibur_rep_c100012_1145	5B	695659830	0.00066788	3.18
HI_T2	GLM	Excalibur_rep_c100012_1145	5B	695659830	0.00044471	3.36
CHL_C	FarmCPU	BS00065401_51	5D	230944800	0.00055733	3.26
CHL_T2	FarmCPU	BS00064691_51	5D	496067069	0.00040475	3.4
CHL_T2	GLM	BS00064691_51	5D	496067069	5.61E-05	4.26
CHL_T2	GLM	RAC875_c14078_1788	5D	561705425	0.00052573	3.28
CHL_T2	MLM	BS00064691_51	5D	496067069	0.00099245	3.01
FLA_C	GLM	IAAV2542	5D	434566554	0.00022293	3.66
FLA_T1	FarmCPU	RAC875_c5518_1401	5D	74464487	5.90E-05	4.23
FLA_T1	GLM	RAC875_c5518_1401	5D	74464487	5.35E-05	4.28
FLA_T1	MLM	RAC875_c5518_1401	5D	74464487	0.00024955	3.61
GpS_T1	GLM	BS00082423_51	5D	489775856	0.00041808	3.38

GY_T2	GLM	RAC875_rep_c101430_180	5D	143783093	0.00024638	3.61
NDVI_T1	FarmCPU	RAC875_c8100_163	5D	560503263	0.00017934	3.75
NDVI_T1	FarmCPU	wsnp_Ex_c11055_17928283	5D	561705358	0.00033918	3.47
NDVI_T1	GLM	RAC875_c8100_163	5D	560503263	0.00034401	3.47
NDVI_T1	GLM	wsnp_Ex_c11055_17928283	5D	561705358	0.00079774	3.1
NDVI_T1	MLM	RAC875_c8100_163	5D	560503263	0.00056986	3.25
NDVI_T1	MLM	wsnp_Ex_c11055_17928283	5D	561705358	0.00092547	3.04
PH_T2	FarmCPU	BS00024761_51	5D	439787057	0.00017111	3.77
PH_T2	FarmCPU	BS00093588_51	5D	440647564	0.00017111	3.77
PH_T2	FarmCPU	RAC875_rep_c89232_502	5D	440903125	0.00017111	3.77
PH_T2	GLM	BS00024761_51	5D	439787057	0.0002268	3.65
PH_T2	GLM	BS00093588_51	5D	440647564	0.0002268	3.65
PH_T2	GLM	RAC875_rep_c89232_502	5D	440903125	0.0002268	3.65
PH_T2	MLM	BS00024761_51	5D	439787057	0.00061337	3.22
PH_T2	MLM	BS00093588_51	5D	440647564	0.00061337	3.22
PH_T2	MLM	RAC875_rep_c89232_502	5D	440903125	0.00061337	3.22
BM_C	FarmCPU	BobWhite_c27364_296	6A	613988559	0.00029169	3.54
BM_C	GLM	RFL_Contig2765_1148	6A	604885062	0.00070201	3.16
BM_C	GLM	BobWhite_c27364_296	6A	613988559	0.00012966	3.89
BM_C	MLM	BobWhite_c27364_296	6A	613988559	0.00082848	3.09
BM_T1	GLM	RAC875_c1998_1744	6A	599050895	0.00067787	3.17
BM_T2	FarmCPU	RAC875_rep_c106371_205	6A	23723234	0.00050367	3.3
CHL_C	FarmCPU	BobWhite_c4255_127	6A	210950123	0.00030516	3.52
CHL_C	GLM	BobWhite_c4255_127	6A	210950123	0.00038983	3.41
CHL_C	MLM	BobWhite_c4255_127	6A	210950123	0.00080689	3.1
FLA_C	FarmCPU	BS00010408_51	6A	612107188	0.00022611	3.65
FLA_C	FarmCPU	Ex_c4507_1299	6A	612108040	0.00021165	3.68
FLA_C	FarmCPU	RAC875_c21938_1408	6A	614803952	0.00020179	3.7
FLA_C	GLM	BS00010408_51	6A	612107188	0.00064546	3.2
FLA_C	GLM	Ex_c4507_1299	6A	612108040	0.00051814	3.29
FLA_C	GLM	RAC875_c21938_1408	6A	614803952	0.00074933	3.13
FLA_C	MLM	BS00010408_51	6A	612107188	0.00082516	3.09
FLA_C	MLM	Ex_c4507_1299	6A	612108040	0.00078985	3.11
FLA_T1	FarmCPU	Excalibur_c37474_242	6A	553858451	0.00063494	3.2
FLA_T1	GLM	Excalibur_c37474_242	6A	553858451	0.00058516	3.24
GpS_T1	FarmCPU	RAC875_c35430_439	6A	614791223	0.00064892	3.19
GpS_T1	GLM	Ku_c69999_111	6A	552549745	0.000894	3.05
GpS_T1	GLM	RAC875_c35430_439	6A	614791223	0.00098671	3.01
GY_C	GLM	BS00063175_51	6A	479890412	0.00081625	3.09
GY_T2	FarmCPU	Ku_c9204_918	6A	217630318	0.0009029	3.05
NDVI_C	FarmCPU	RAC875_c23552_1354	6A	15748045	0.00087234	3.06
NDVI_C	FarmCPU	RAC875_c77113_57	6A	581334759	0.00047586	3.33
NDVI_C	FarmCPU	wsnp_Ra_c2270_4383252	6A	595610691	0.00072149	3.15
NDVI_C	FarmCPU	BS00099074_51	6A	595627657	0.00024348	3.62
NDVI_C	FarmCPU	BS00003185_51	6A	596165625	0.0007315	3.14
NDVI_C	GLM	RAC875_c23552_1354	6A	15748045	0.00055177	3.26

NDVI_C	GLM	RAC875_c77113_57	6A	581334759	0.00085216	3.07
NDVI_C	GLM	BS00099074_51	6A	595627657	0.00048211	3.32
NDVI_C	MLM	BS00099074_51	6A	595627657	0.00085849	3.07
NDVI_T1	FarmCPU	BS00109919_51	6A	4678836	0.000586	3.24
NDVI_T1	FarmCPU	RAC875_c7804_236	6A	447833393	0.00029047	3.54
NDVI_T1	FarmCPU	IACX2250	6A	456752340	0.00046568	3.34
NDVI_T1	GLM	BS00109919_51	6A	4678836	0.00096529	3.02
NDVI_T1	GLM	RAC875_c7804_236	6A	447833393	0.00083239	3.08
NDVI_T1	GLM	IACX2250	6A	456752340	0.00084064	3.08
NDVI_T1	MLM	RAC875_c7804_236	6A	447833393	0.00086089	3.07
NDVI_T2	GLM	IAAV1385	6A	388213861	0.00035801	3.45
NDVI_T2	GLM	BS00078715_51	6A	424093800	0.00035801	3.45
NDVI_T2	GLM	Excalibur_c34574_452	6A	449693203	0.0003934	3.41
NDVI_T2	GLM	IAAV7384	6A	454649474	0.0003934	3.41
NDVI_T2	GLM	BS00004466_51	6A	599035144	0.0007636	3.12
PH_T2	FarmCPU	wsnp_Ex_c48789_53586406	6A	550074242	0.00084732	3.08
PH_T2	GLM	wsnp_Ex_c48789_53586406	6A	550074242	0.00095299	3.03
T.P_C	GLM	Tdurum_contig29629_437	6A	550738341	0.00081354	3.09
T.P_C	GLM	Ex_c24379_1031	6A	550955015	0.00081354	3.09
T.P_C	GLM	Ra_c11721_766	6A	550955740	0.00081354	3.09
T.P_C	GLM	Ku_c56003_719	6A	550958366	0.00081354	3.09
T.P_C	GLM	Ku_c21399_772	6A	550960495	0.00081354	3.09
T.p_T1	FarmCPU	RAC875_c5893_368	6A	611855600	0.00056839	3.25
T.p_T1	GLM	RAC875_c5893_368	6A	611855600	0.00075826	3.13
T.p_T2	FarmCPU	wsnp_Ex_c51820_55631560	6A	565468100	0.0004935	3.31
T.p_T2	FarmCPU	IAAV4068	6A	565468131	0.00057019	3.25
T.p_T2	FarmCPU	BobWhite_c13845_195	6A	567933315	0.00011408	3.95
T.p_T2	FarmCPU	IAAV5761	6A	567933533	0.00019187	3.72
T.p_T2	FarmCPU	IAAV8730	6A	568494476	0.0004935	3.31
T.p_T2	FarmCPU	BS00082640_51	6A	568504960	0.00031469	3.51
T.p_T2	FarmCPU	BobWhite_c32372_186	6A	568506977	0.00011408	3.95
T.p_T2	FarmCPU	wsnp_Ku_c14219_22455933	6A	569120165	0.00019187	3.72
T.p_T2	FarmCPU	wsnp_Ex_rep_c70951_69806211	6A	569122657	0.00076723	3.12
T.p_T2	FarmCPU	wsnp_Ex_rep_c70951_69806455	6A	569123854	0.00076038	3.12
T.p_T2	GLM	BobWhite_c13845_195	6A	567933315	0.00036483	3.44
T.p_T2	GLM	IAAV5761	6A	567933533	0.00063772	3.2
T.p_T2	GLM	BS00082640_51	6A	568504960	0.00075929	3.12
T.p_T2	GLM	BobWhite_c32372_186	6A	568506977	0.00036483	3.44
T.p_T2	GLM	wsnp_Ku_c14219_22455933	6A	569120165	0.00063772	3.2
T.p_T2	MLM	BobWhite_c13845_195	6A	567933315	0.00064875	3.19
T.p_T2	MLM	BobWhite_c32372_186	6A	568506977	0.00064875	3.19
HI_C	FarmCPU	BobWhite_c19647_159	6A	189576934	0.00030649	3.52
HI_C	GLM	BobWhite_c19647_159	6A	189576934	2.92E-05	4.54
HI_C	GLM	Tdurum_contig53138_302	6A	454649324	0.0008834	3.06
HI_C	MLM	BobWhite_c19647_159	6A	189576934	0.0008073	3.1
HI_T1	GLM	wsnp_Ku_c3450_6387847	6A	545832350	0.0009847	3.01

HI_T1	GLM	CAP8_c6448_265	6A	604882756	0.00070011	3.16
HI_T2	GLM	wsnp_CAP12_rep_c4048_1842112	6A	606768042	0.0005035	3.3
NAE_T2	FarmCPU	Ku_c21490_472	6A	307683151	0.00072885	3.14
NAE_T2	FarmCPU	BS00036397_51	6A	543941017	0.000405	3.4
NAE_T2	FarmCPU	IAAV1652	6A	544204977	0.00079452	3.1
NAE_T2	FarmCPU	wsnp_Ex_c11348_18327861	6A	544205496	0.00055633	3.26
NAE_T2	FarmCPU	wsnp_Ex_c11348_18326787	6A	544206570	0.00031177	3.51
NAE_T2	FarmCPU	wsnp_Ex_c9502_15748251	6A	544476235	0.00090207	3.05
NAE_T2	FarmCPU	Tdurum_contig42125_5972	6A	545828799	1.53E-05	4.82
NAE_T2	FarmCPU	BobWhite_c1082_134	6A	548411545	0.0001584	3.81
NAE_T2	FarmCPU	BobWhite_c19820_129	6A	548419425	0.0004224	3.38
NAE_T2	FarmCPU	IAAV4703	6A	549036170	5.08E-05	4.3
NAE_T2	GLM	Ku_c21490_472	6A	307683151	3.98E-05	4.41
NAE_T2	GLM	Kukri_c24790_253	6A	358598949	0.0003071	3.52
NAE_T2	GLM	wsnp_Ku_c12588_20290369	6A	379657961	0.0003071	3.52
NAE_T2	GLM	BS00064462_51	6A	457073554	0.00096911	3.02
NAE_T2	GLM	BS00036397_51	6A	543941017	0.00028912	3.54
NAE_T2	GLM	Excalibur_c11578_324	6A	544151540	0.00077663	3.11
NAE_T2	GLM	IAAV1652	6A	544204977	0.0008603	3.07
NAE_T2	GLM	wsnp_Ex_c11348_18327861	6A	544205496	0.00043344	3.37
NAE_T2	GLM	wsnp_Ex_c11348_18326787	6A	544206570	0.00027138	3.57
NAE_T2	GLM	wsnp_Ex_c9502_15748251	6A	544476235	0.00052315	3.29
NAE_T2	GLM	Tdurum_contig42125_5972	6A	545828799	5.75E-06	5.25
NAE_T2	GLM	Excalibur_c26057_1049	6A	547739803	0.00037831	3.43
NAE_T2	GLM	BobWhite_c1082_134	6A	548411545	3.41E-05	4.47
NAE_T2	GLM	BobWhite_c19820_129	6A	548419425	0.00010281	3.99
NAE_T2	GLM	IAAV4703	6A	549036170	1.53E-05	4.82
NAE_T2	MLM	Tdurum_contig42125_5972	6A	545828799	0.00013213	3.88
NAE_T2	MLM	BobWhite_c1082_134	6A	548411545	0.00060709	3.22
NAE_T2	MLM	IAAV4703	6A	549036170	0.00028214	3.55
BM_C	FarmCPU	Excalibur_c130_3813	6B	713513400	0.00021142	3.68
BM_C	FarmCPU	wsnp_Ku_c46363_53116979	6B	712500073	0.00021372	3.68
BM_C	FarmCPU	BobWhite_c27364_124	6B	713971271	0.00029169	3.54
BM_C	GLM	wsnp_Ku_c46363_53116979	6B	712500073	0.00020701	3.69
BM_C	GLM	Excalibur_c130_3813	6B	713513400	0.00020734	3.69
BM_C	GLM	BobWhite_c27364_124	6B	713971271	0.00012966	3.89
BM_C	MLM	wsnp_Ku_c46363_53116979	6B	712500073	0.0006722	3.18
BM_C	MLM	Excalibur_c130_3813	6B	713513400	0.00066498	3.18
BM_C	MLM	BobWhite_c27364_124	6B	713971271	0.00082848	3.09
CHL_C	FarmCPU	wsnp_Ex_c1383_2651887	6B	681316926	0.00024963	3.61
CHL_C	FarmCPU	Kukri_c75359_152	6B	681317076	2.46E-05	4.61
CHL_C	FarmCPU	wsnp_Ex_c1383_2652398	6B	681317437	0.00077406	3.12
CHL_C	GLM	RAC875_c4420_371	6B	519151149	0.00060411	3.22
CHL_C	GLM	Excalibur_c7785_123	6B	526481222	0.00056681	3.25
CHL_C	GLM	wsnp_Ra_c46591_52408053	6B	571734783	0.00069199	3.16
CHL_C	GLM	wsnp_Ex_c1383_2651887	6B	681316926	0.00040727	3.4

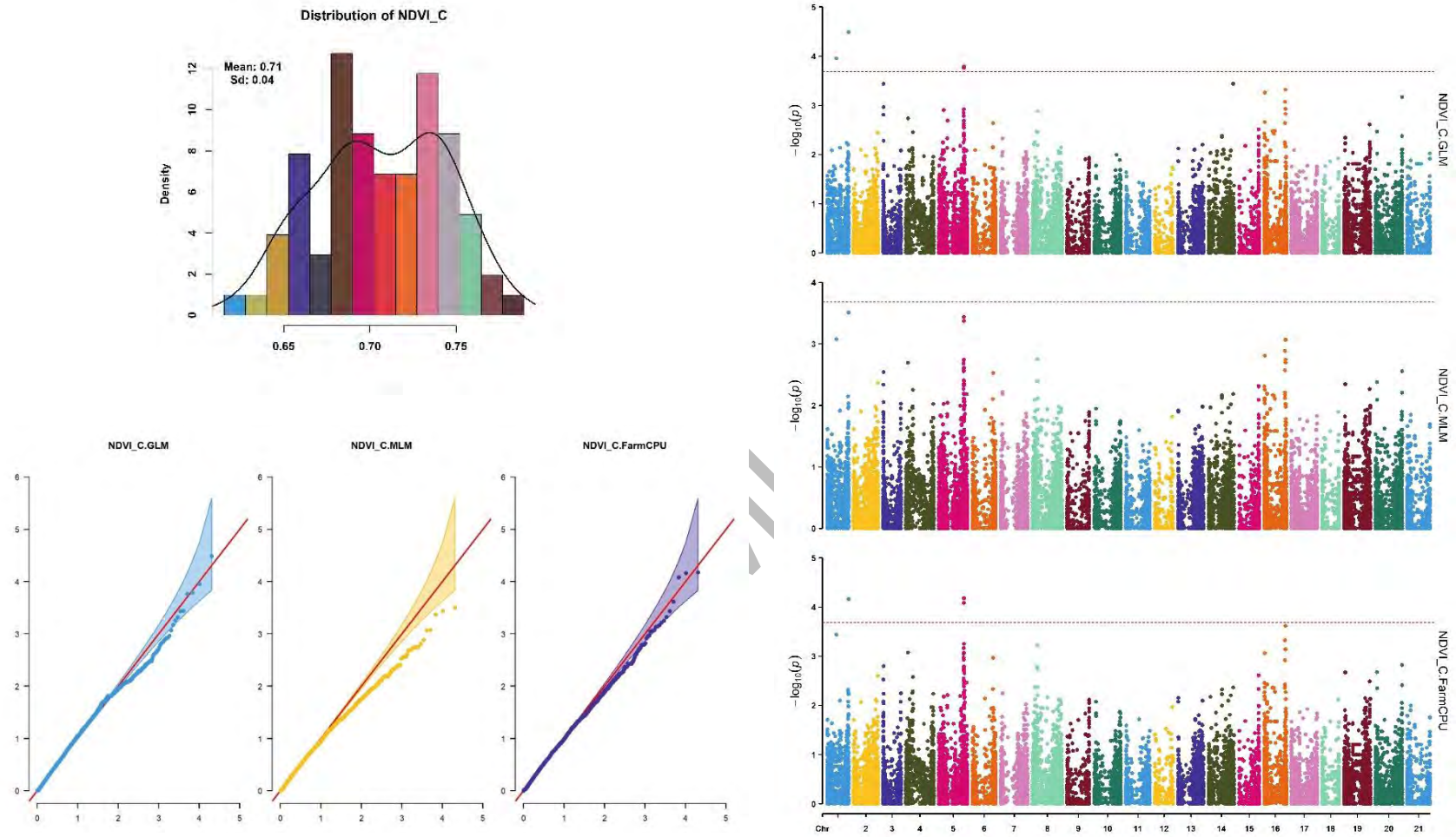
CHL_C	GLM	Kukri_c75359_152	6B	681317076	4.43E-05	4.36
CHL_C	MLM	wsnp_Ex_c1383_2651887	6B	681316926	0.00057582	3.24
CHL_C	MLM	Kukri_c75359_152	6B	681317076	0.00015014	3.83
CHL_T1	MLM	Jagger_c10642_260	6B	1615167	0.00069067	3.17
CHL_T1	FarmCPU	Jagger_c10642_260	6B	1615167	0.0001957	3.71
CHL_T1	FarmCPU	CAP7_rep_c12573_126	6B	709218399	0.00088434	3.06
CHL_T1	FarmCPU	RAC875_c5413_1237	6B	710006969	0.00071037	3.15
CHL_T1	GLM	Jagger_c10642_260	6B	1615167	0.00045581	3.35
FLA_C	FarmCPU	Tdurum_contig55744_822	6B	709532701	0.00033005	3.49
FLA_C	FarmCPU	BS00022240_51	6B	715724322	0.0007433	3.13
FLA_C	FarmCPU	Tdurum_contig28247_226	6B	717861700	0.00024678	3.61
FLA_C	FarmCPU	Tdurum_contig65998_258	6B	717967303	0.00020861	3.69
FLA_C	FarmCPU	BS00069822_51	6B	718231415	0.00052917	3.28
FLA_C	FarmCPU	BS00110383_51	6B	718232151	9.62E-05	4.02
FLA_C	FarmCPU	BobWhite_c43263_180	6B	718920710	0.00015439	3.82
FLA_C	FarmCPU	BobWhite_c13202_399	6B	720506966	0.00017745	3.76
FLA_C	FarmCPU	RAC875_c57219_1439	6B	720507303	6.97E-05	4.16
FLA_C	FarmCPU	BS00074151_51	6B	720759233	0.0006757	3.18
FLA_C	GLM	Tdurum_contig55744_822	6B	709532701	0.0009129	3.04
FLA_C	GLM	Tdurum_contig28247_226	6B	717861700	0.00046511	3.34
FLA_C	GLM	Tdurum_contig65998_258	6B	717967303	0.00050702	3.3
FLA_C	GLM	BS00110383_51	6B	718232151	0.00030746	3.52
FLA_C	GLM	BobWhite_c43263_180	6B	718920710	0.00036985	3.44
FLA_C	GLM	BobWhite_c13202_399	6B	720506966	0.00064775	3.19
FLA_C	GLM	RAC875_c57219_1439	6B	720507303	0.00030006	3.53
FLA_C	MLM	Tdurum_contig65998_258	6B	717967303	0.00084554	3.08
FLA_C	MLM	BS00110383_51	6B	718232151	0.00064858	3.19
FLA_C	MLM	BobWhite_c43263_180	6B	718920710	0.00087724	3.06
FLA_C	MLM	BobWhite_c13202_399	6B	720506966	0.00068888	3.17
FLA_C	MLM	RAC875_c57219_1439	6B	720507303	0.00050295	3.3
GpS_C	GLM	Excalibur_c2049_2593	6B	717916435	0.00087613	3.06
GpS_T1	FarmCPU	Tdurum_contig47204_301	6B	42393676	0.00064892	3.19
GpS_T1	FarmCPU	BS00098103_51	6B	115700466	0.00062139	3.21
GpS_T1	FarmCPU	TA002465-0455-w	6B	115701090	0.00063338	3.2
GpS_T1	FarmCPU	BS00023021_51	6B	717961357	0.00064892	3.19
GpS_T1	FarmCPU	BS00110651_51	6B	718232004	0.00064892	3.19
GpS_T1	FarmCPU	BS00065783_51	6B	720408098	0.00064892	3.19
GpS_T1	GLM	Tdurum_contig47204_301	6B	42393676	0.00098671	3.01
GpS_T1	GLM	BS00023021_51	6B	717961357	0.00098671	3.01
GpS_T1	GLM	BS00110651_51	6B	718232004	0.00098671	3.01
GpS_T1	GLM	BS00065783_51	6B	720408098	0.00098671	3.01
GpS_T2	GLM	Tdurum_contig43538_1687	6B	3889272	0.0001461	3.84
GpS_T2	GLM	wsnp_Ku_c2119_4098330	6B	8410279	0.00058902	3.23
GY_C	FarmCPU	BS00046263_51	6B	704974467	0.00024923	3.61
GY_C	GLM	BS00046263_51	6B	704974467	7.80E-05	4.11
GY_C	MLM	BS00046263_51	6B	704974467	0.0008722	3.06

GY_T1	FarmCPU	Tdurum_contig29294_171	6B	461265920	0.00019844	3.71
GY_T1	GLM	Tdurum_contig29294_171	6B	461265920	0.00026426	3.58
GY_T1	MLM	Tdurum_contig29294_171	6B	461265920	0.00058743	3.24
GY_T2	FarmCPU	RAC875_c17847_123	6B	705384526	4.71E-06	5.33
GY_T2	GLM	RAC875_c17847_123	6B	705384526	0.00040187	3.4
GY_T2	GLM	BS00034339_51	6B	705553527	0.00040187	3.4
GY_T2	MLM	RAC875_c17847_123	6B	705384526	0.00072379	3.15
GY_T2	MLM	BS00034339_51	6B	705553527	0.00072379	3.15
NDVI_T1	FarmCPU	BS00064967_51	6B	706332736	0.00015461	3.82
NDVI_T1	FarmCPU	Tdurum_contig55744_822	6B	709532701	0.00010627	3.98
NDVI_T1	FarmCPU	RAC875_c5413_1237	6B	710006969	3.82E-05	4.42
NDVI_T1	FarmCPU	RAC875_c5413_1266	6B	710006998	0.00051686	3.29
NDVI_T1	GLM	BS00064967_51	6B	706332736	0.00039117	3.41
NDVI_T1	GLM	Tdurum_contig55744_822	6B	709532701	0.00027206	3.57
NDVI_T1	GLM	RAC875_c5413_1237	6B	710006969	0.00011002	3.96
NDVI_T1	GLM	RAC875_c5413_1266	6B	710006998	0.00069779	3.16
NDVI_T1	MLM	BS00064967_51	6B	706332736	0.00055111	3.26
NDVI_T1	MLM	Tdurum_contig55744_822	6B	709532701	0.00042828	3.37
NDVI_T1	MLM	RAC875_c5413_1237	6B	710006969	0.00021664	3.67
NDVI_T2	FarmCPU	BS00033642_51	6B	26650950	0.00030417	3.52
NDVI_T2	GLM	BS00033642_51	6B	26650950	8.12E-05	4.1
NDVI_T2	MLM	BS00033642_51	6B	26650950	0.00099574	3.01
PH_C	FarmCPU	Excalibur_c2049_323	6B	717910496	0.00085285	3.07
T.p_T2	FarmCPU	BS00108381_51	6B	130829948	0.00023934	3.63
T.p_T2	GLM	BS00010657_51	6B	4490367	0.00069917	3.16
T.p_T2	GLM	BS00108381_51	6B	130829948	0.00029054	3.54
T.p_T2	MLM	BS00108381_51	6B	130829948	0.00084236	3.08
HI_T1	FarmCPU	Jagger_c555_287	6B	191991803	0.00066068	3.19
HI_T1	GLM	GENE-4221_519	6B	661341226	0.00049783	3.31
HI_T1	MLM	GENE-4221_519	6B	661341226	0.00039413	3.41
HI_T2	FarmCPU	Kukri_rep_c104521_727	6B	634332344	0.0009085	3.05
HI_T2	FarmCPU	Kukri_rep_c104521_117	6B	634333515	0.00082054	3.09
HI_T2	FarmCPU	RAC875_c4030_1310	6B	705790058	0.00056465	3.25
NAE_T2	FarmCPU	BS00111086_51	6B	41703780	0.00054928	3.27
NAE_T2	FarmCPU	RAC875_rep_c107929_341	6B	41704461	0.00087506	3.06
NAE_T2	GLM	BS00111086_51	6B	41703780	0.00040374	3.4
FLA_C	FarmCPU	Tdurum_contig10729_64	6D	470317575	1.64E-06	5.79
FLA_C	GLM	Tdurum_contig10729_64	6D	470317575	7.31E-06	5.14
FLA_C	MLM	Tdurum_contig10729_64	6D	470317575	4.27E-05	4.37
GY_T2	GLM	Kukri_c9310_156	6D	4282077	0.00067855	3.17
GY_T2	GLM	Excalibur_c46335_294	6D	463446233	0.0009063	3.05
HI_C	FarmCPU	IAAV2245	6D	221850822	0.0005134	3.29
HI_C	GLM	IAAV2245	6D	221850822	0.00061585	3.22
HI_T1	GLM	GENE-4221_94	6D	437167074	0.00049783	3.31
HI_T1	GLM	IACX1609	6D	437177936	0.00049783	3.31
HI_T1	MLM	GENE-4221_94	6D	437167074	0.00039413	3.41

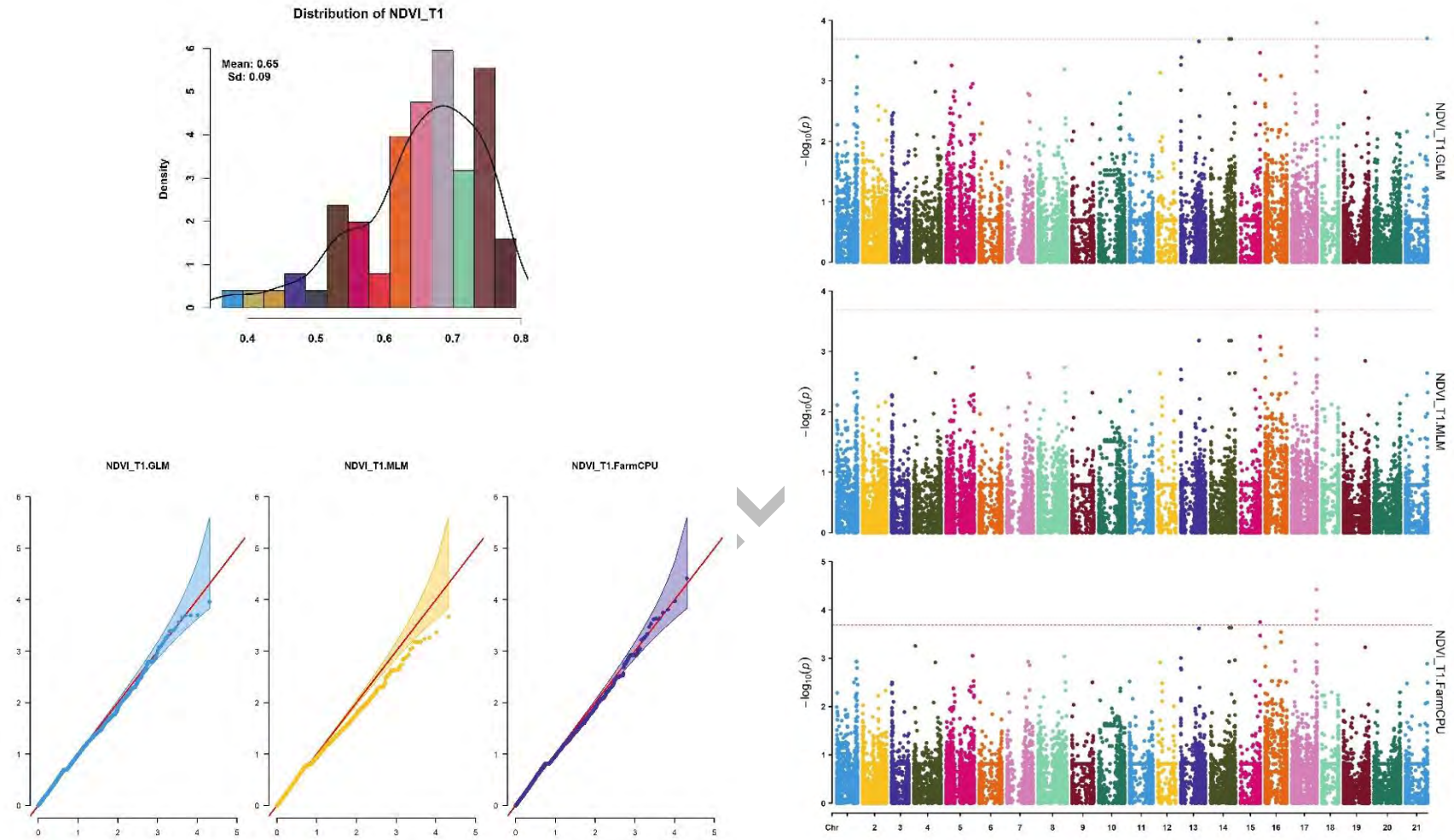
HI_T1	MLM	IACX1609	6D	437177936	0.00039413	3.41
BM_C	FarmCPU	Kukri_rep_c101179_404	7A	12309482	0.00079417	3.11
BM_C	FarmCPU	Excalibur_c1421_358	7A	6542668	0.00090779	3.05
BM_C	GLM	Excalibur_c1421_358	7A	6542668	0.00095009	3.03
BM_C	GLM	Kukri_rep_c101179_404	7A	12309482	0.00097716	3.02
CHL_C	FarmCPU	Kukri_c36926_201	7A	534401	0.00032214	3.5
CHL_C	FarmCPU	Ku_c1006_729	7A	5080402	0.00078114	3.11
CHL_C	FarmCPU	CAP7_c3756_190	7A	501588178	0.00072704	3.14
CHL_C	FarmCPU	wsnp_Ra_c16287_24904962	7A	610208884	0.00085269	3.07
CHL_C	GLM	Kukri_c36926_201	7A	534401	0.00017841	3.75
CHL_C	GLM	CAP7_c3756_190	7A	501588178	0.00030218	3.52
CHL_C	MLM	Kukri_c36926_201	7A	534401	0.00071225	3.15
CHL_C	MLM	Ku_c1006_729	7A	5080402	0.0007061	3.16
FLA_C	GLM	wsnp_Ku_c11530_18803034	7A	731268522	0.00022999	3.64
FLA_T1	FarmCPU	wsnp_Ra_rep_c105182_89171305	7A	585066140	0.00030552	3.52
FLA_T1	FarmCPU	Kukri_rep_c105157_485	7A	611333656	0.00037114	3.44
FLA_T1	GLM	RAC875_rep_c105182_460	7A	585066042	0.00064849	3.19
FLA_T1	GLM	wsnp_Ra_rep_c105182_89171305	7A	585066140	0.00010732	3.97
FLA_T1	GLM	wsnp_Ex_c49880_54354165	7A	585066192	0.00064849	3.19
FLA_T1	GLM	Kukri_rep_c105157_485	7A	611333656	0.00013662	3.87
FLA_T1	GLM	BS00065529_51	7A	689260096	0.00028972	3.54
FLA_T1	MLM	wsnp_Ra_rep_c105182_89171305	7A	585066140	0.00080568	3.1
FLA_T1	MLM	Kukri_rep_c105157_485	7A	611333656	0.00093069	3.04
GpS_T1	GLM	wsnp_Ex_c21068_30195276	7A	182619306	0.00081119	3.1
GY_C	FarmCPU	BS00003726_51	7A	112265455	0.00075842	3.13
GY_C	GLM	BS00003726_51	7A	112265455	0.00050687	3.3
NDVI_T1	FarmCPU	wsnp_Ku_c5160_9203226	7A	626897816	0.00059711	3.23
NDVI_T2	FarmCPU	BS00022751_51	7A	159557364	0.00049764	3.31
NDVI_T2	FarmCPU	BS00065077_51	7A	162521335	0.00089244	3.05
NDVI_T2	FarmCPU	BobWhite_c24063_231	7A	232746065	0.00088854	3.06
NDVI_T2	GLM	BS00022751_51	7A	159557364	0.00057165	3.25
NDVI_T2	GLM	BS00065077_51	7A	162521335	0.00075593	3.13
NDVI_T2	GLM	Kukri_c39894_178	7A	232593162	0.00095726	3.02
NDVI_T2	GLM	BobWhite_c24063_231	7A	232746065	0.00095089	3.03
NDVI_T2	GLM	IAAV5328	7A	236620290	0.00095726	3.02
PH_C	FarmCPU	Tdurum_contig92906_272	7A	19817000	0.00082114	3.09
PH_C	FarmCPU	Kukri_c76470_79	7A	19955881	0.00082114	3.09
T.P_C	FarmCPU	Excalibur_c20311_388	7A	1371537	0.00068439	3.17
T.p_T1	FarmCPU	Kukri_rep_c105330_552	7A	140994002	0.00042527	3.38
T.p_T1	FarmCPU	Kukri_rep_c75743_357	7A	141857745	0.0003894	3.41
T.p_T1	GLM	Kukri_rep_c105330_552	7A	140994002	0.00015269	3.82
T.p_T1	GLM	Kukri_rep_c75743_357	7A	141857745	0.00015632	3.81
T.p_T1	MLM	Kukri_rep_c75743_357	7A	141857745	0.00096448	3.02
T.p_T2	GLM	BS00023003_51	7A	364242892	0.00023588	3.63
HI_C	GLM	BS00065529_51	7A	689260096	0.00092738	3.04
HI_T1	FarmCPU	wsnp_Ra_c12773_20367106	7A	731269487	0.00033448	3.48

HI_T1	GLM	BS00067564_51	7A	46906967	0.00021634	3.67
HI_T1	GLM	BS00082180_51	7A	120176416	0.00051845	3.29
HI_T1	GLM	Kukri_c10197_186	7A	205456514	0.00066929	3.18
HI_T1	GLM	IAAV5805	7A	205456515	0.00066929	3.18
HI_T1	GLM	IAAV6170	7A	538784427	0.00021634	3.67
HI_T1	MLM	BS00067564_51	7A	46906967	0.00026766	3.58
HI_T1	MLM	IAAV6170	7A	538784427	0.00026766	3.58
HI_T2	FarmCPU	BS00036553_51	7A	32011341	0.0002306	3.64
HI_T2	FarmCPU	RAC875_c4732_1672	7A	573432281	0.00098748	3.01
HI_T2	GLM	Excalibur_c22708_566	7A	8802871	0.00093053	3.04
HI_T2	GLM	BS00036553_51	7A	32011341	0.00053883	3.27
HI_T2	GLM	Jagger_c10704_106	7A	709885363	0.00071132	3.15
HI_T2	MLM	BS00036553_51	7A	32011341	0.00065538	3.19
BM_T1	FarmCPU	wsnp_Ku_c665_1371448	7B	58727983	0.00098758	3.01
BM_T1	FarmCPU	Ku_c665_985	7B	58728043	0.00049944	3.31
BM_T1	GLM	Ku_c665_985	7B	58728043	0.00053426	3.28
CHL_C	FarmCPU	Kukri_c20180_112	7B	347335040	0.00055733	3.26
FLA_T1	FarmCPU	Excalibur_c25630_537	7B	666325389	0.00078823	3.11
FLA_T1	GLM	Excalibur_c25630_537	7B	666325389	0.00060905	3.22
FLA_T2	FarmCPU	wsnp_Ex_c17882_26646153	7B	68344442	0.00068065	3.17
FLA_T2	FarmCPU	BS00076675_51	7B	653289313	0.00024648	3.61
FLA_T2	GLM	Tdurum_contig11827_678	7B	5056846	0.00025957	3.59
FLA_T2	GLM	wsnp_Ex_c17882_26646153	7B	68344442	0.00044856	3.35
FLA_T2	GLM	BS00076675_51	7B	653289313	4.42E-05	4.36
FLA_T2	MLM	BS00076675_51	7B	653289313	0.00068787	3.17
GpS_T2	FarmCPU	Tdurum_contig43954_1287	7B	701187687	0.0001105	3.96
GpS_T2	FarmCPU	IAAV3313	7B	701187837	0.0001105	3.96
GpS_T2	FarmCPU	BS00108264_51	7B	701212480	0.00062843	3.21
GpS_T2	FarmCPU	BobWhite_c5046_372	7B	701219250	0.00055505	3.26
GpS_T2	FarmCPU	Ku_c9679_453	7B	703166486	0.00044382	3.36
GpS_T2	FarmCPU	Ra_c35421_250	7B	704270157	0.0005232	3.29
GpS_T2	GLM	Tdurum_contig43954_1287	7B	701187687	0.00011454	3.95
GpS_T2	GLM	IAAV3313	7B	701187837	0.00011454	3.95
GpS_T2	GLM	Tdurum_contig43954_2291	7B	701188949	0.00097015	3.02
GpS_T2	GLM	BobWhite_c5046_372	7B	701219250	0.00066954	3.18
GpS_T2	GLM	Ku_c9679_453	7B	703166486	0.00039823	3.4
GpS_T2	GLM	Ra_c35421_250	7B	704270157	0.00071017	3.15
GpS_T2	GLM	RFL_Contig5898_807	7B	706861606	0.00035337	3.46
GpS_T2	GLM	BS00110528_51	7B	712072772	0.00052341	3.29
GpS_T2	MLM	Tdurum_contig43954_1287	7B	701187687	0.00038652	3.42
GpS_T2	MLM	IAAV3313	7B	701187837	0.00038652	3.42
GY_C	FarmCPU	BobWhite_c10448_80	7B	66979391	0.00050484	3.3
GY_C	GLM	BobWhite_c10448_80	7B	66979391	0.00029945	3.53
GY_T2	FarmCPU	Kukri_c34272_108	7B	187428763	0.00039186	3.41
GY_T2	GLM	Tdurum_contig27385_131	7B	148218463	0.00087276	3.06
GY_T2	GLM	Excalibur_rep_c116278_53	7B	221262445	0.00084801	3.08

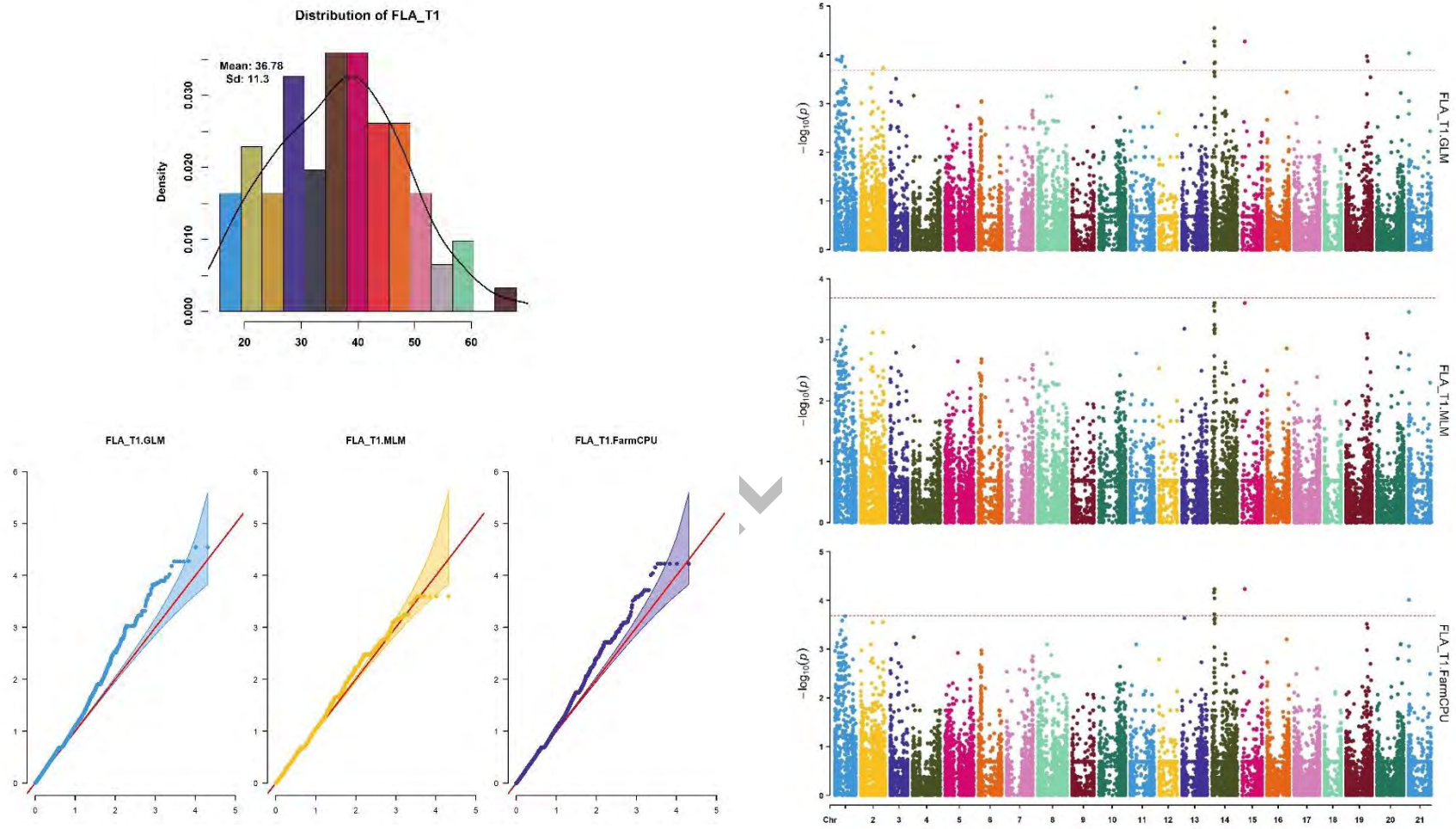
GY_T2	GLM	RAC875_c967_355	7B	226611888	0.00024638	3.61
NDVI_C	GLM	RAC875_c1329_298	7B	744604731	0.00067669	3.17
PH_T2	FarmCPU	Ra_c26852_957	7B	700830514	0.00010422	3.99
PH_T2	GLM	Ra_c26852_957	7B	700830514	0.00017299	3.77
PH_T2	MLM	Ra_c26852_957	7B	700830514	0.00049563	3.31
HI_T1	GLM	BS00076402_51	7B	3794526	0.00066523	3.18
HI_T1	GLM	Excalibur_c60612_236	7B	5056454	0.00098644	3.01
HI_T1	MLM	BS00076402_51	7B	3794526	0.00085863	3.07
HI_T2	FarmCPU	Tdurum_contig15690_413	7B	584317162	0.00031937	3.5
HI_T2	GLM	Tdurum_contig15690_413	7B	584317162	0.00075425	3.13
HI_T2	GLM	Tdurum_contig5083_1164	7B	644438851	0.00049964	3.31
HI_T2	GLM	Ku_c16895_803	7B	645124723	0.0007318	3.14
HI_T2	MLM	Tdurum_contig15690_413	7B	584317162	0.00083227	3.08
FLA_T1	FarmCPU	Ex_c5231_1655	7D	9305754	0.00087281	3.06
FLA_T1	FarmCPU	Ra_c9123_3192	7D	9307439	9.74E-05	4.02
FLA_T1	GLM	Ex_c5231_1655	7D	9305754	0.00090207	3.05
FLA_T1	GLM	Ra_c9123_3192	7D	9307439	9.41E-05	4.03
FLA_T1	MLM	Ra_c9123_3192	7D	9307439	0.00035348	3.46
FLA_T2	FarmCPU	BS00066148_51	7D	550214613	0.00094343	3.03
GpS_T2	FarmCPU	Kukri_rep_c72901_271	7D	611761888	0.00086462	3.07
GpS_T2	GLM	Kukri_rep_c72901_271	7D	611761888	0.00057463	3.25
GY_C	GLM	Kukri_c39812_125	7D	633251828	0.00076169	3.12
GY_C	GLM	D_F5XZDLF01CK3P1_55	7D	633373836	0.00031413	3.51
GY_C	GLM	D_contig65328_393	7D	634592543	0.00035919	3.45
GY_T1	FarmCPU	BS00066389_51	7D	603664675	0.00056803	3.25
GY_T1	GLM	BS00066389_51	7D	603664675	0.00097637	3.02
GY_T2	FarmCPU	Kukri_c11890_709	7D	104902486	1.16E-05	4.94
NDVI_T1	GLM	Kukri_c3781_285	7D	622188273	0.00019932	3.71
NDVI_T2	GLM	GENE-4953_139	7D	220286160	0.00095726	3.02
PH_T2	FarmCPU	Tdurum_contig20965_1446	7D	15223520	0.00030291	3.52
PH_T2	GLM	Tdurum_contig20965_1446	7D	15223520	0.00043315	3.37
PH_T2	GLM	Kukri_c23208_256	7D	182602753	0.00070975	3.15
PH_T2	MLM	Tdurum_contig20965_1446	7D	15223520	0.0009516	3.03
HI_T1	GLM	BobWhite_c2260_168	7D	9310116	0.00021634	3.67
HI_T1	GLM	BobWhite_s63403_99	7D	123049332	0.00051845	3.29
HI_T1	MLM	BobWhite_c2260_168	7D	9310116	0.00026766	3.58



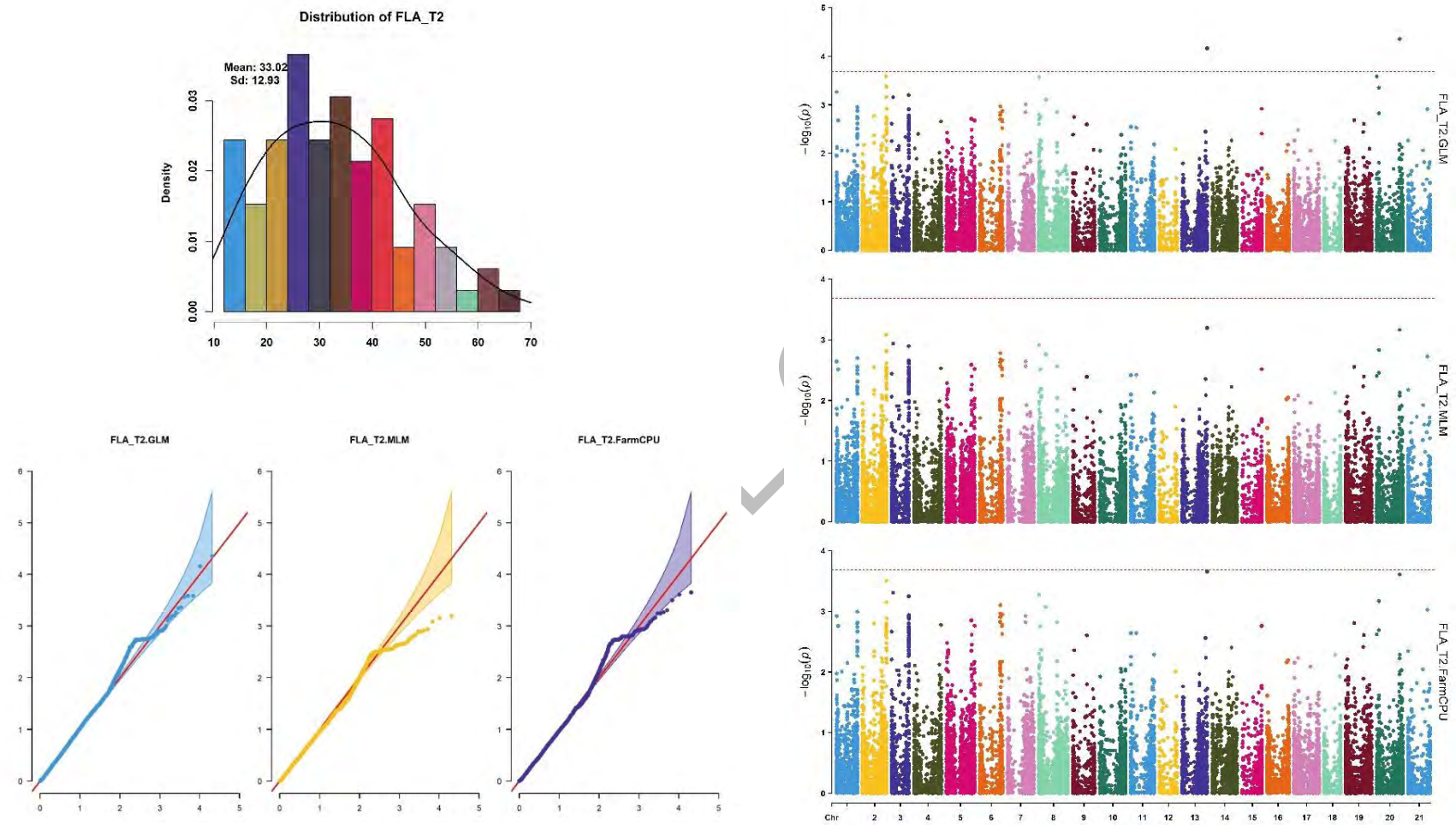
Appendix 2.3. The Density Distribution plot, QQ-plot, and Manhattan plot for NDVI under control; NDVI_C. (a) The density plot is showing the distribution of NDVI_C in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



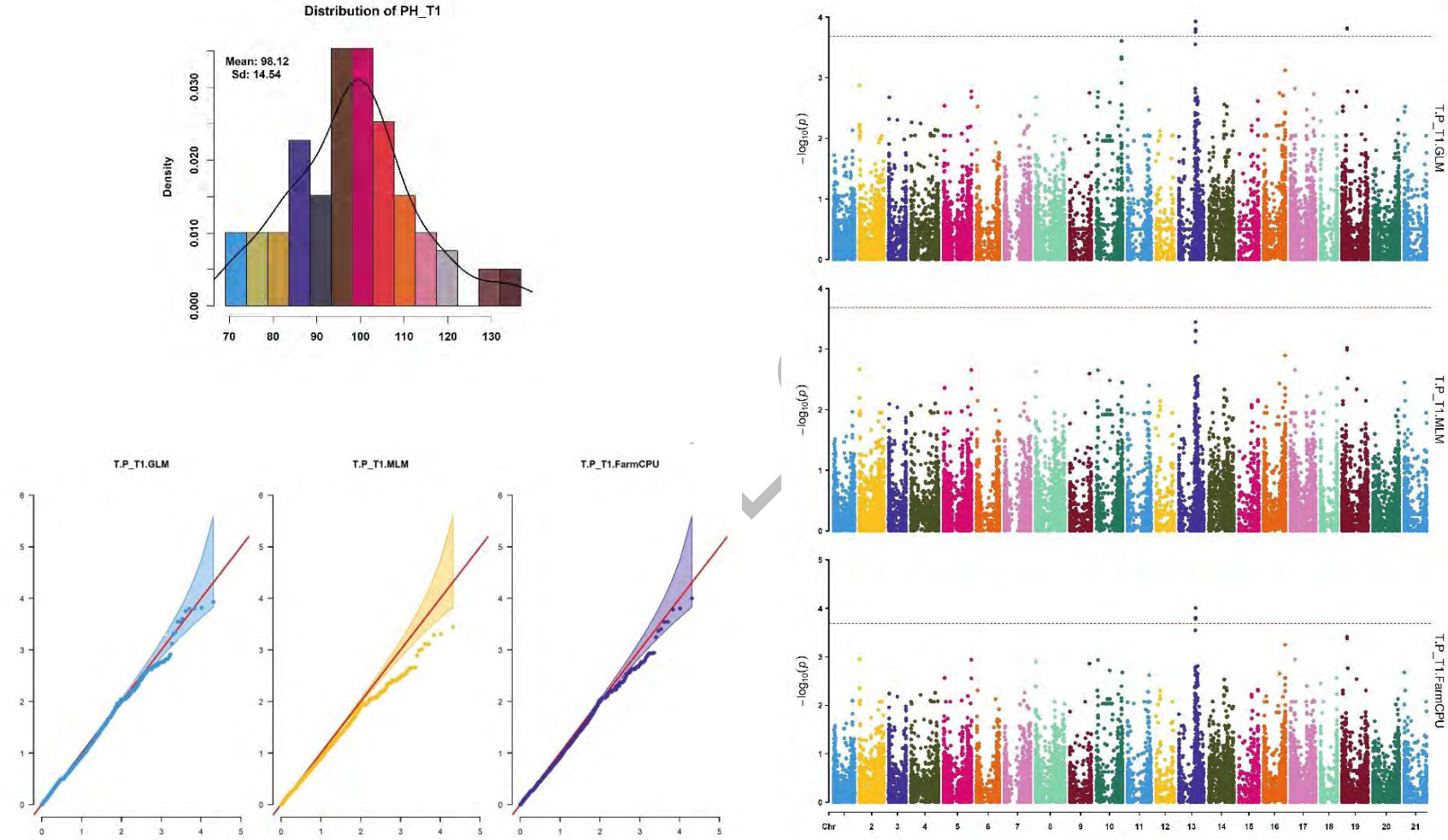
Appendix 2.4. The Density Distribution plot, QQ-plot, and Manhattan plot for NDVI under treatment 1; NDVI_T1. (a) The density plot is showing the distribution of NDVI_T1 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



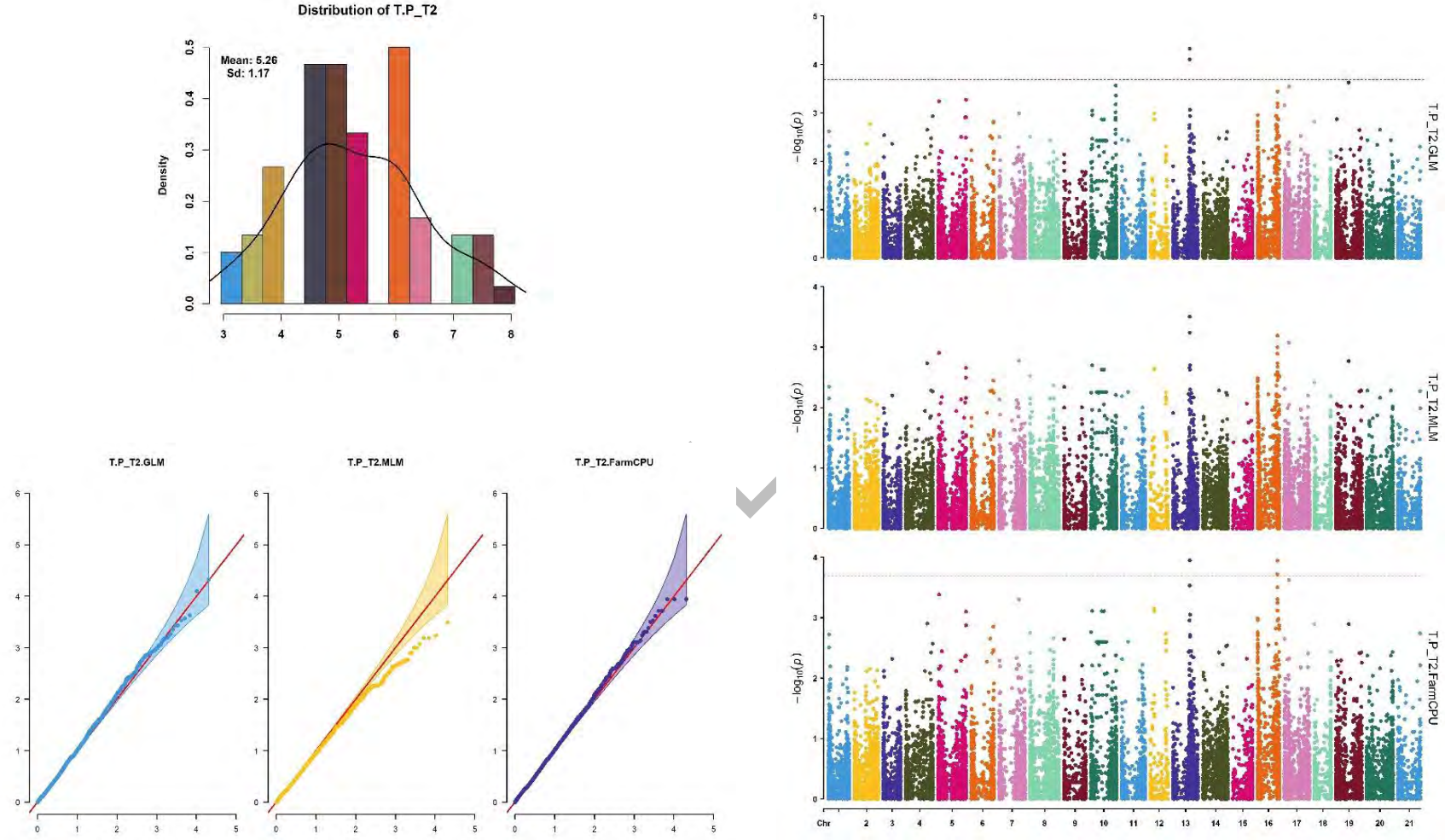
Appendix 2.5. The Density Distribution plot, QQ-plot, and Manhattan plot for flag leaf area under treatment 1; FLA_T1. (a) The density plot is showing the distribution of FLA_T1 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c)Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



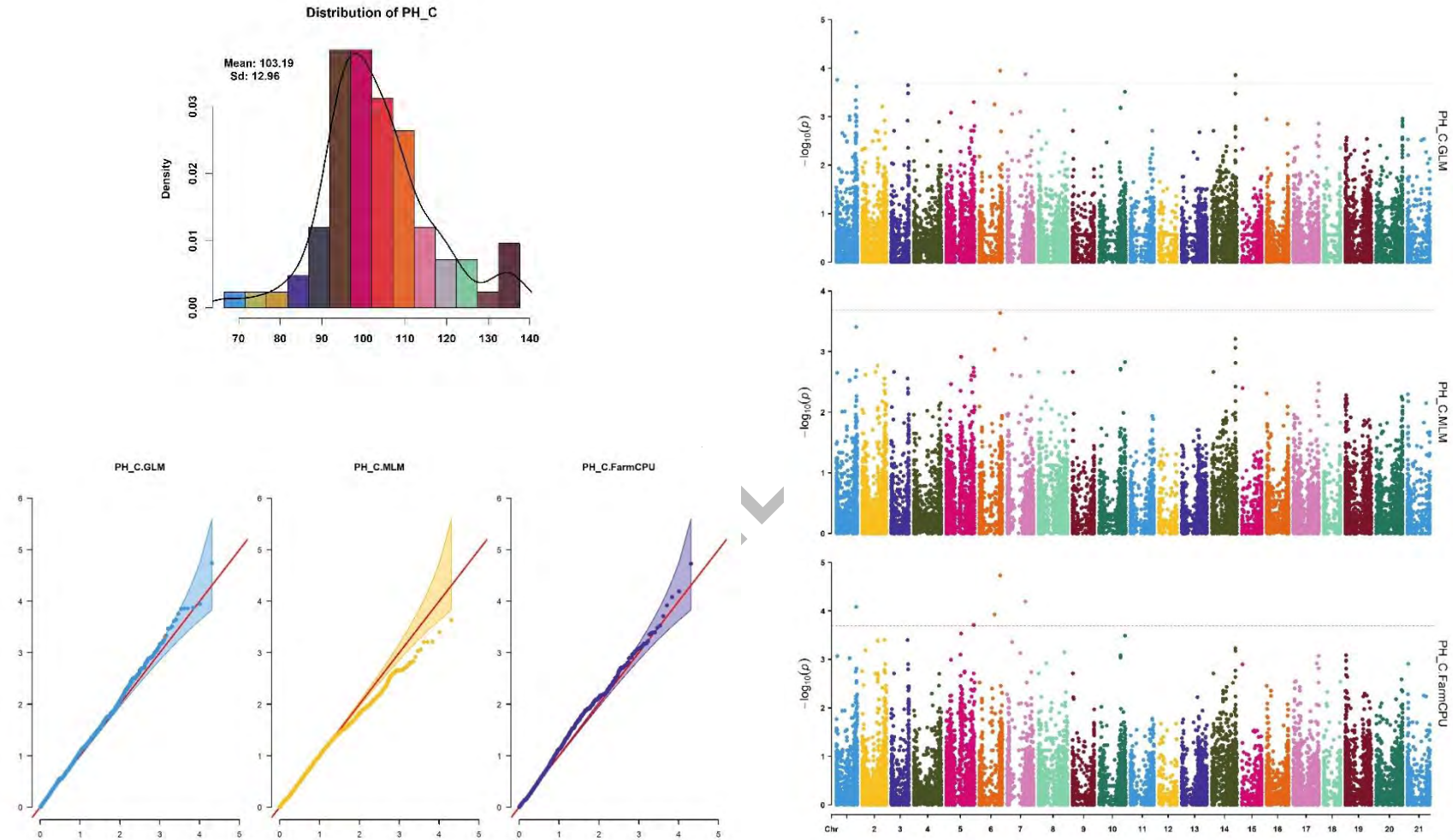
Appendix 2.6. The Density Distribution plot, QQ-plot, and Manhattan plot for flag leaf area under treatment 2; FLA_T2. (a) The density plot is showing the distribution of FLA_T2 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



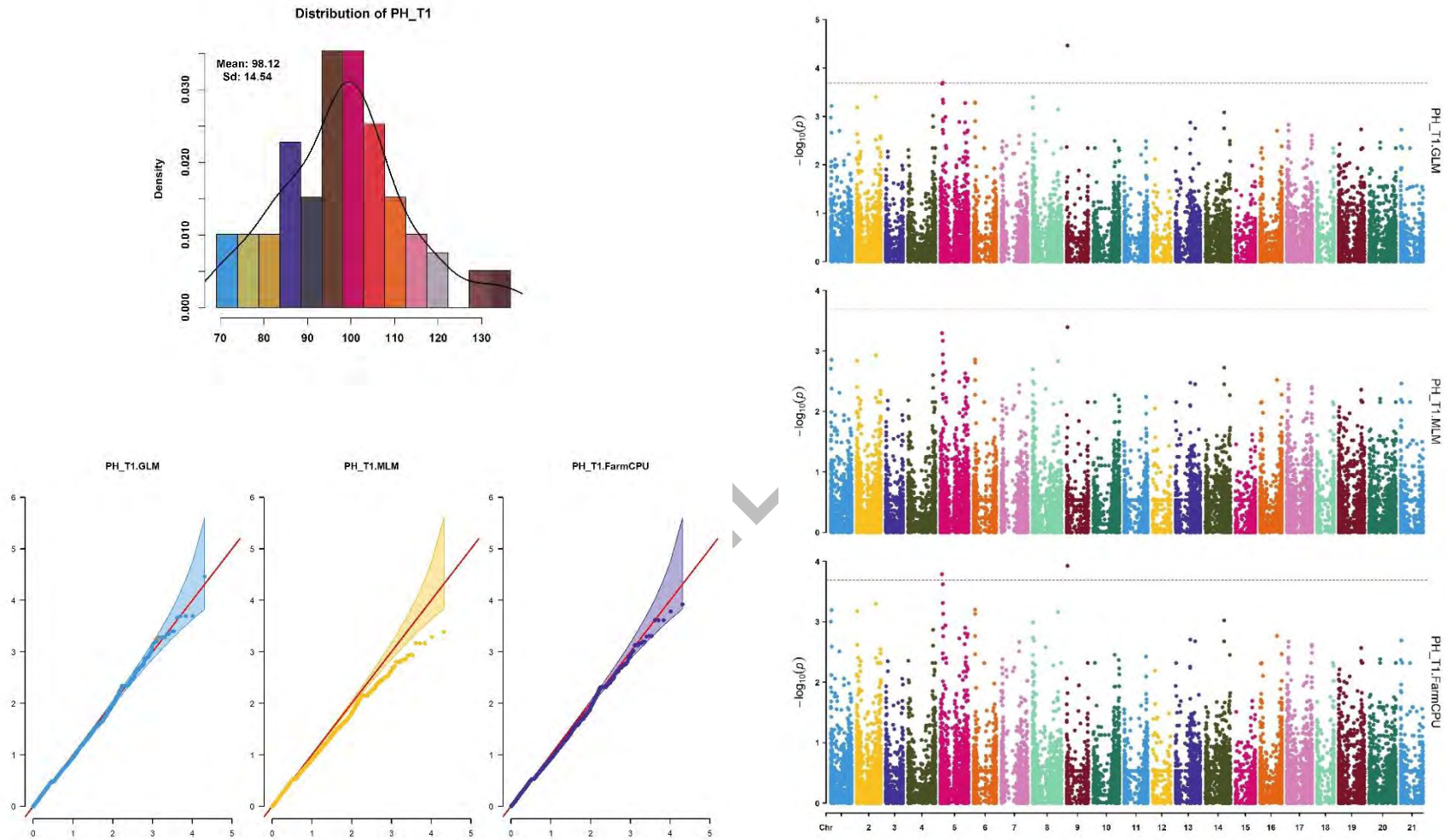
Appendix 2.7. The Density Distribution plot, QQ-plot, and Manhattan plot for tiller per plant under treatment 1; T.P_T1. (a) The density plot is showing the distribution of T.P_T1 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



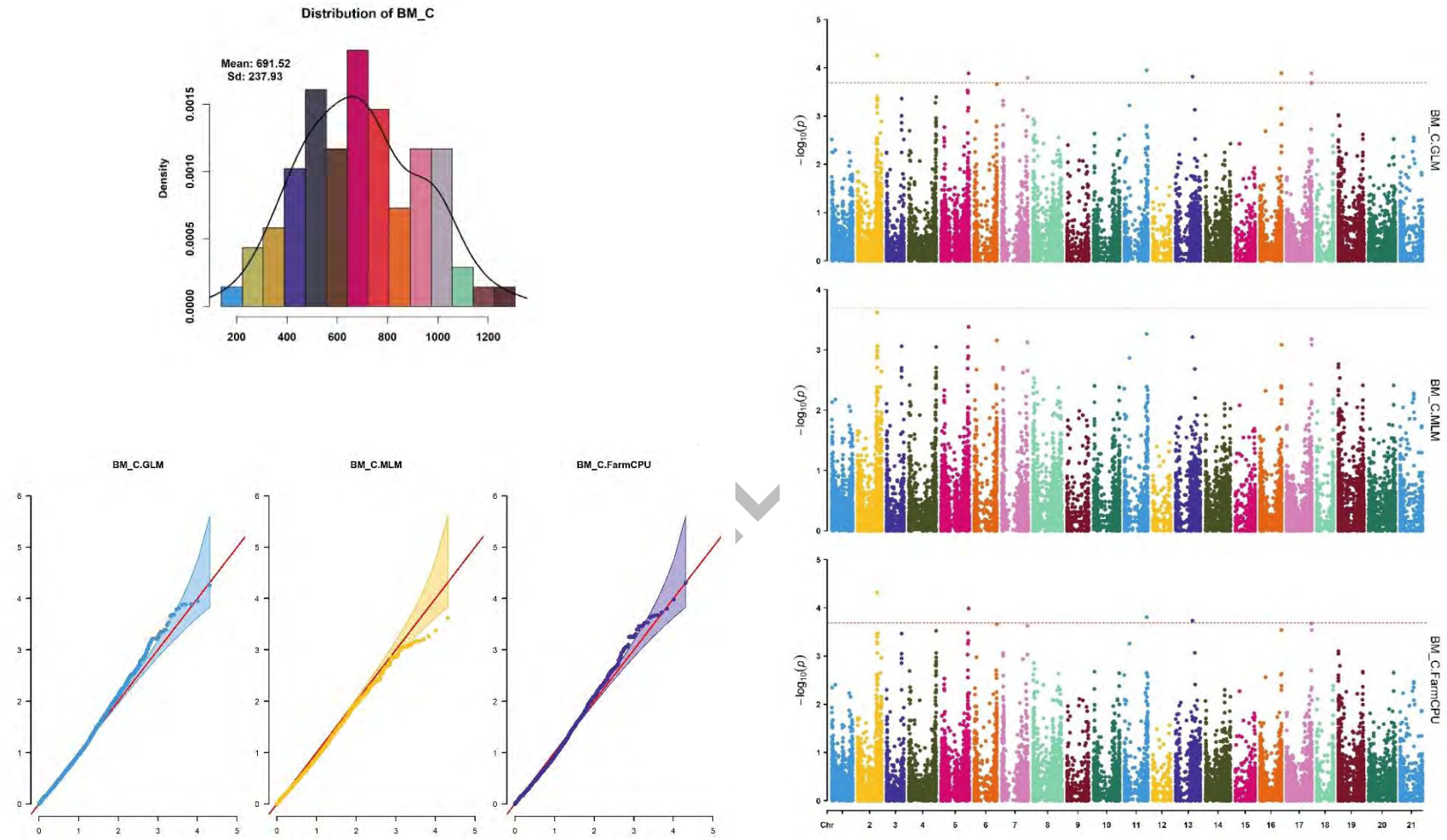
Appendix 2.8. The Density Distribution plot, QQ-plot, and Manhattan plot for tiller per plant under treatment 2; T.P T2. (a) The density plot is showing the distribution of T.P T2 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



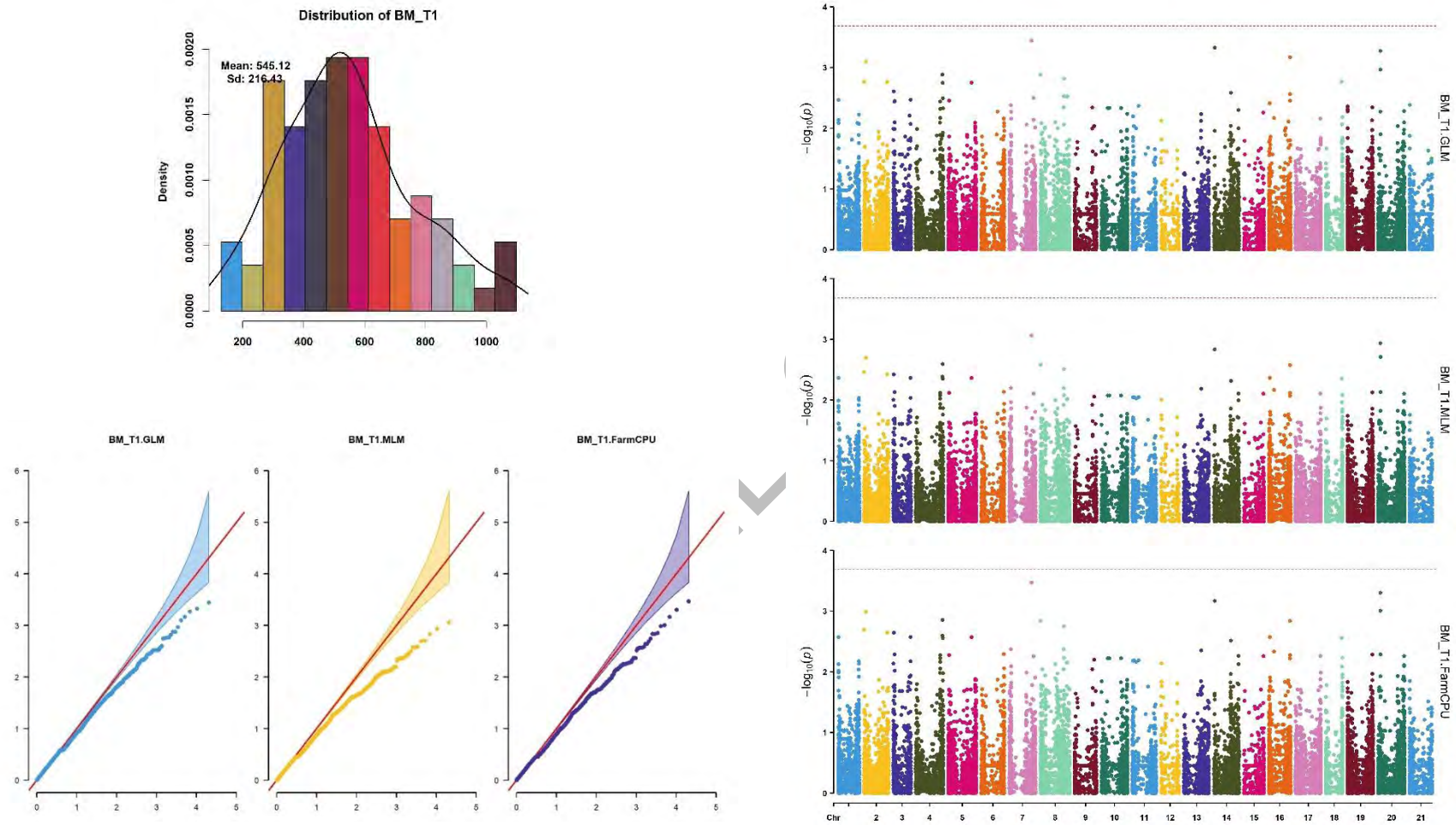
Appendix 2.9. The Density Distribution plot, QQ-plot, and Manhattan plot for plant height under control; PH_C. (a) The density plot is showing the distribution of PH_C in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



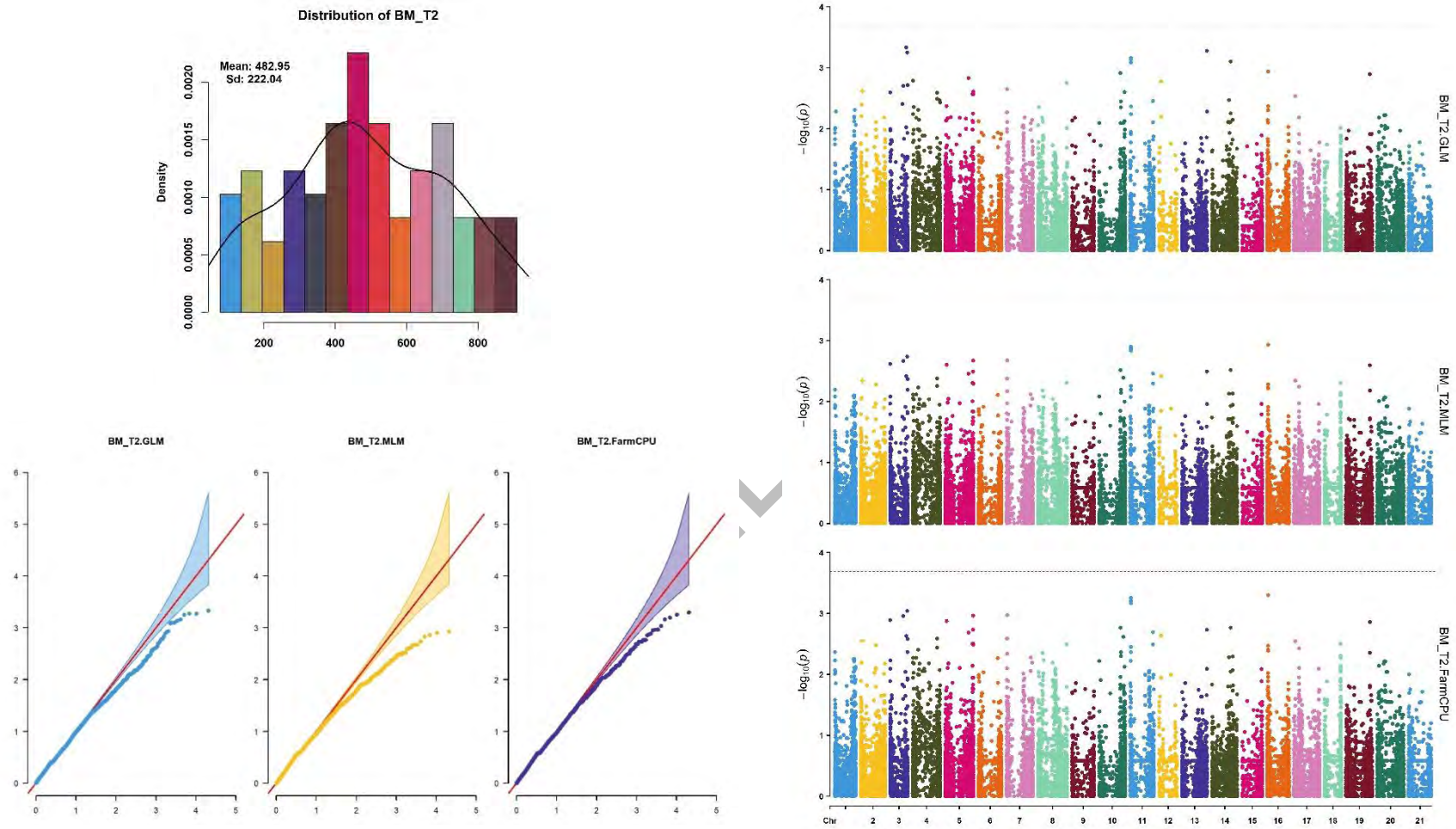
Appendix 2.10. The Density Distribution plot, QQ-plot, and Manhattan plot for plant height under treatment 1; PH_T1. (a) The density plot is showing the distribution of PH_T1 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



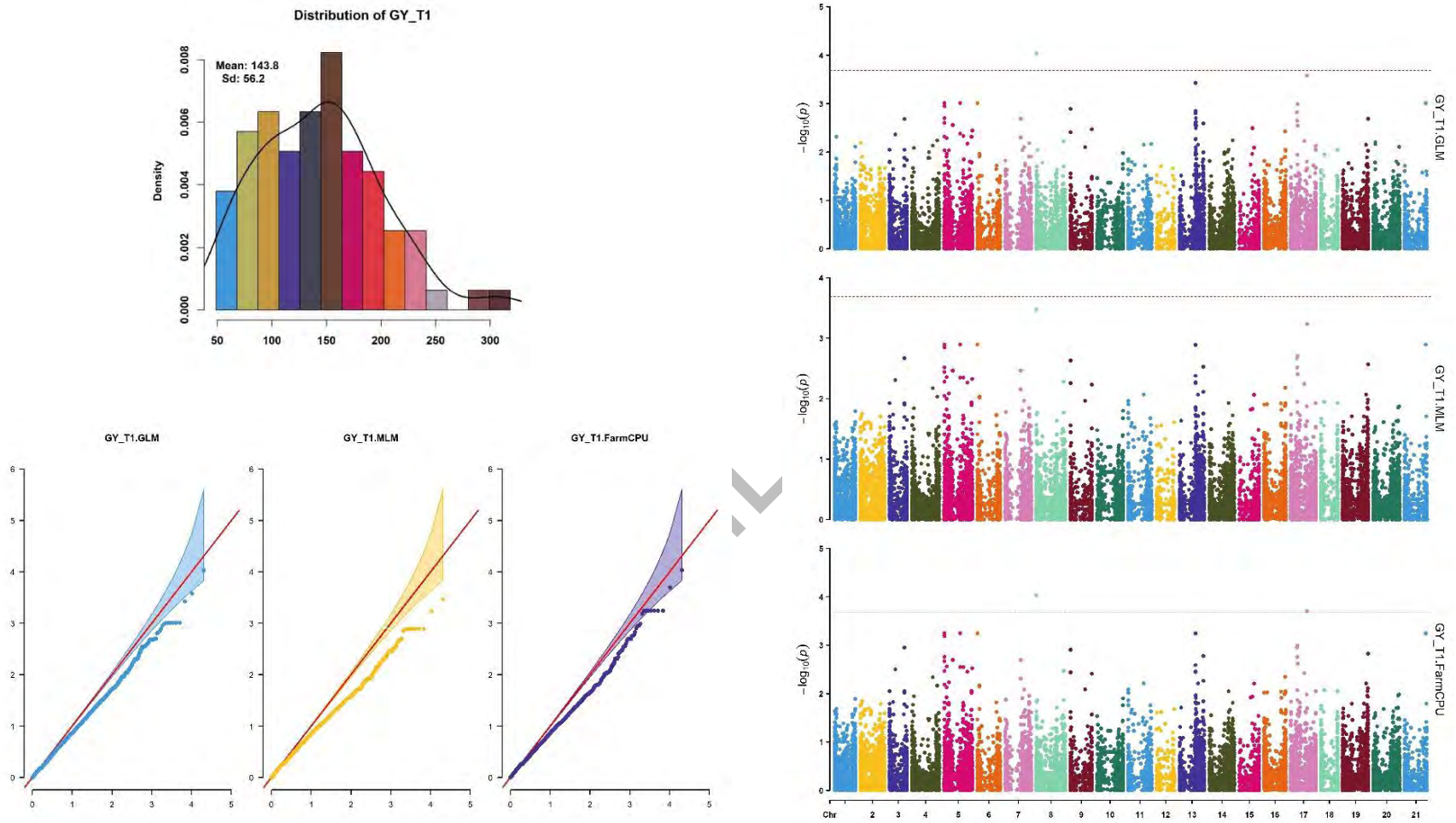
Appendix 2.11. The Density Distribution plot, QQ-plot, and Manhattan plot for biomass under control; BM_C. (a) The density plot is showing the distribution of BM_C in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



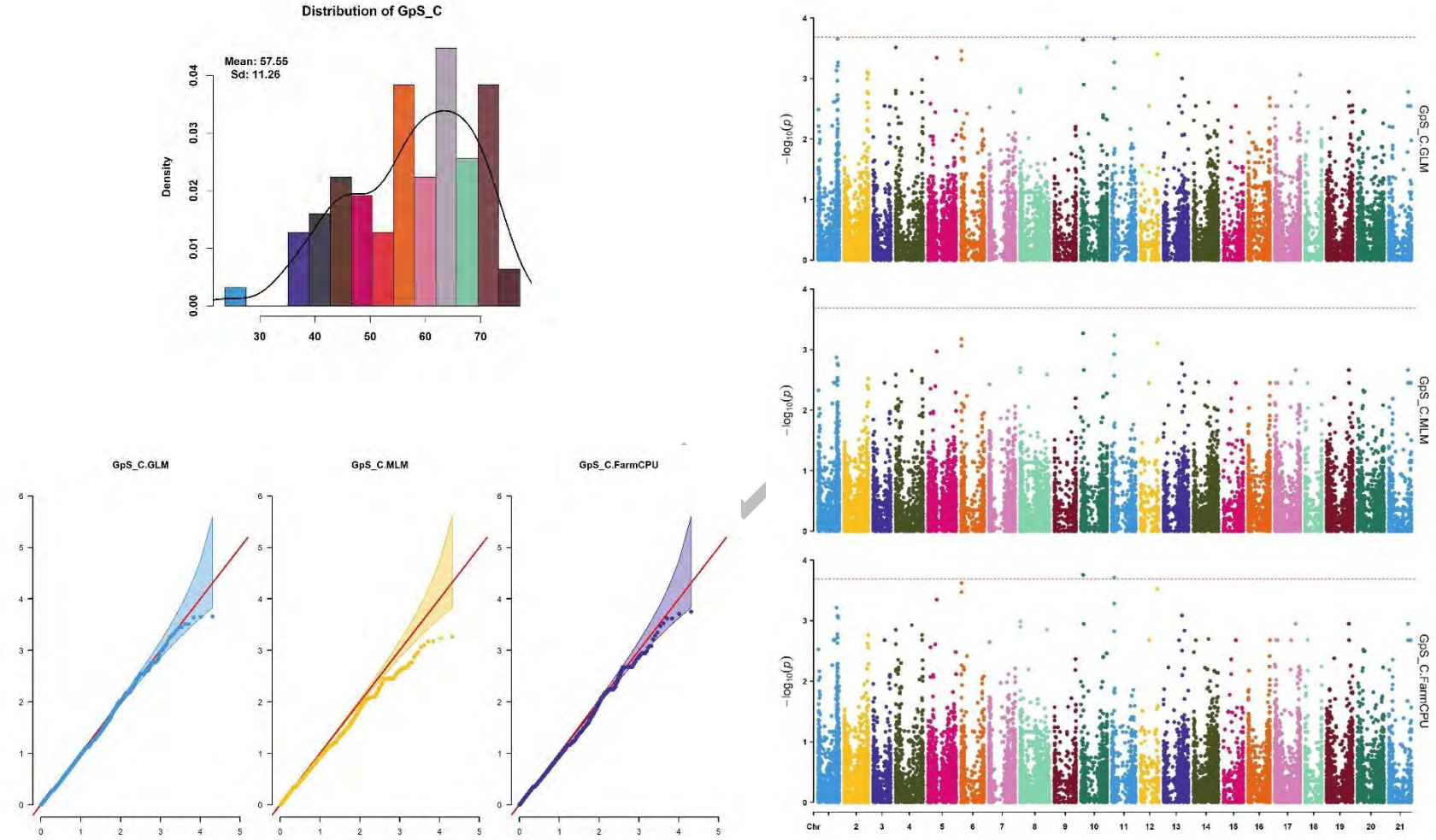
Appendix 2.12. The Density Distribution plot, QQ-plot, and Manhattan plot for biomass under treatment 1; BM_T1. (a) The density plot is showing the distribution of BM_T1 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



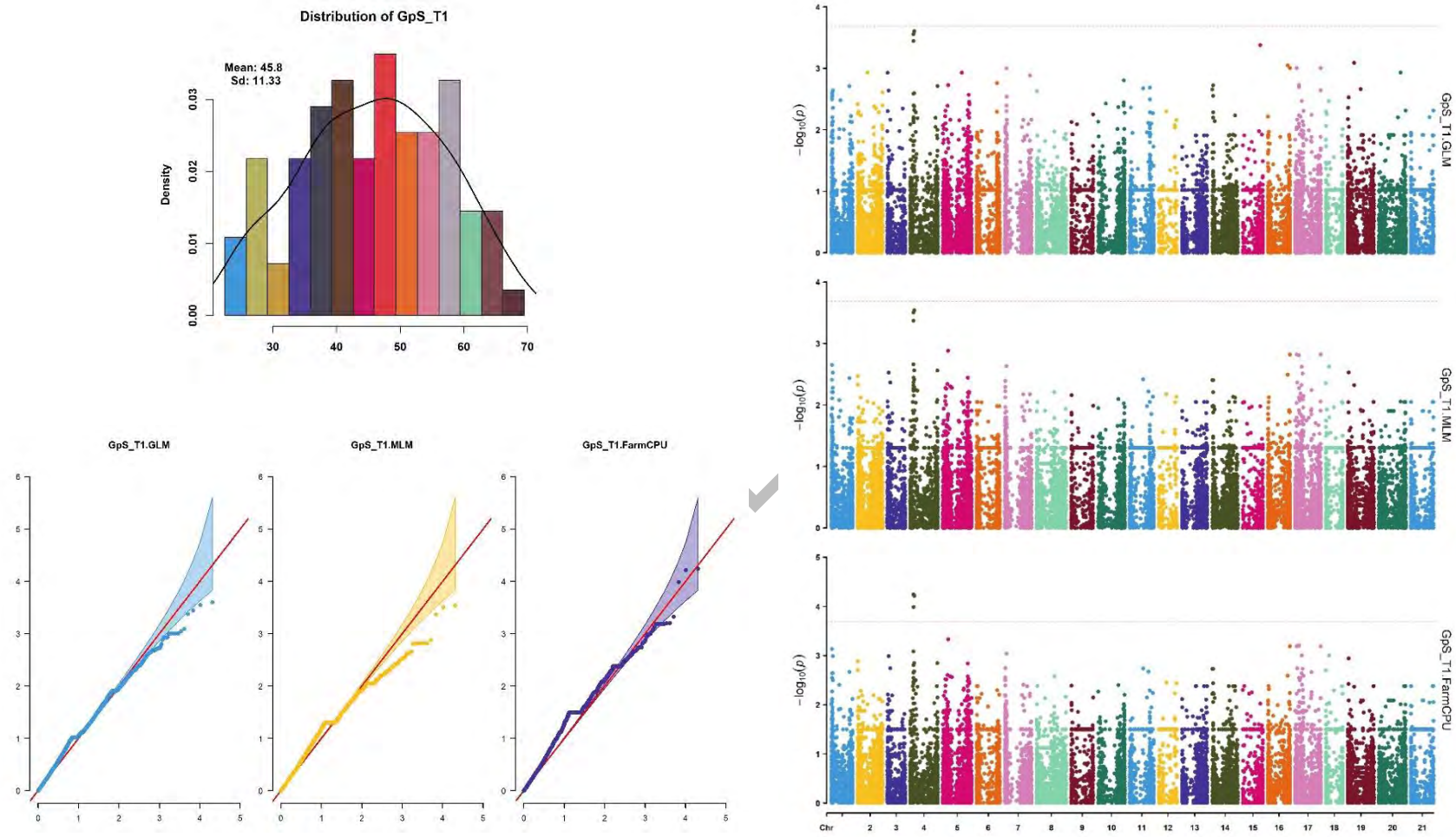
Appendix 2.13. The Density Distribution plot, QQ-plot, and Manhattan plot for biomass under treatment 2; BM_T2. (a) The density plot is showing the distribution of BM_T2 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



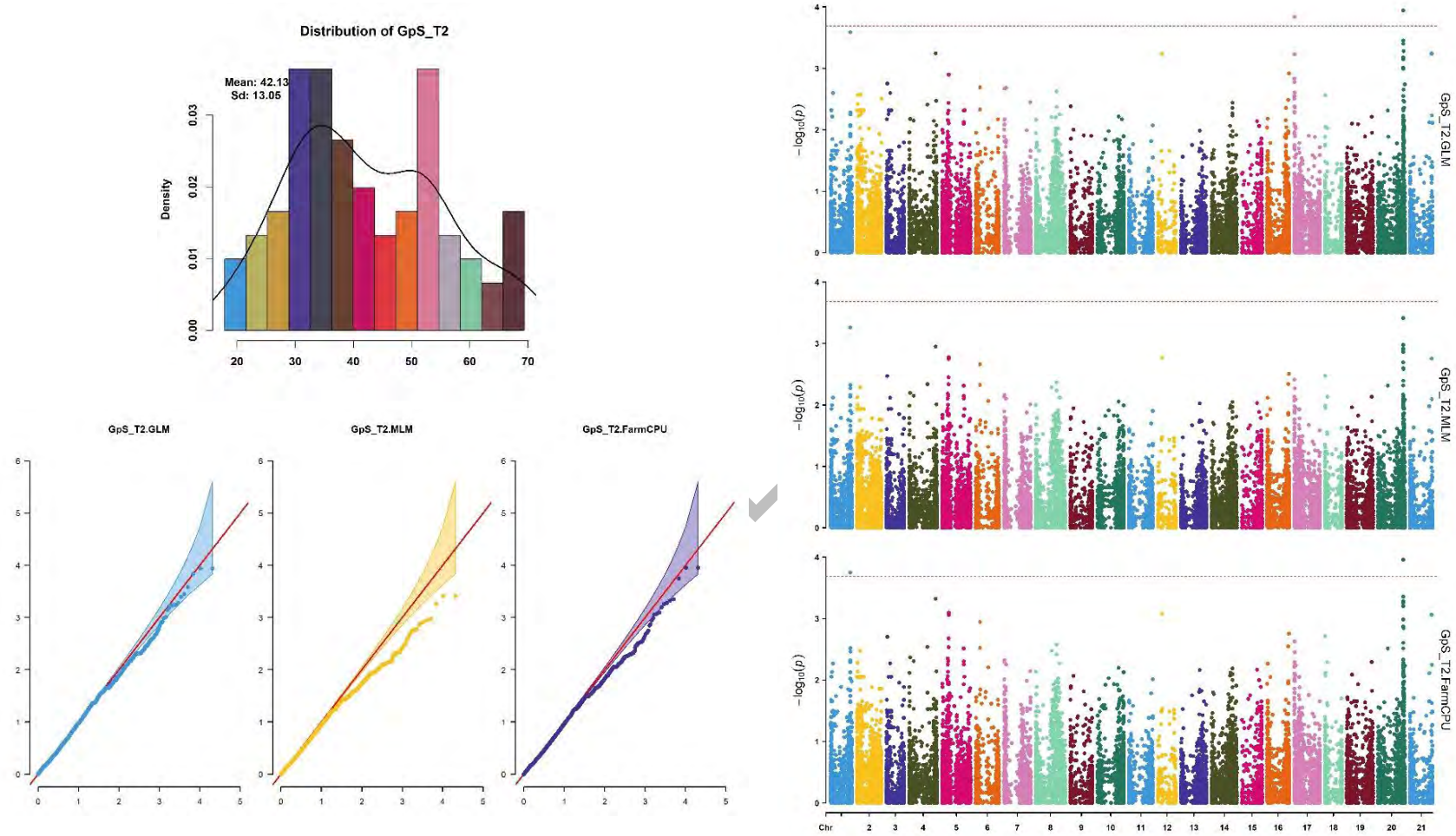
Appendix 2.14. The Density Distribution plot, QQ-plot, and Manhattan plot for grain yield under treatment 1; GY_T1. (a) The density plot is showing the distribution of GY_C in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



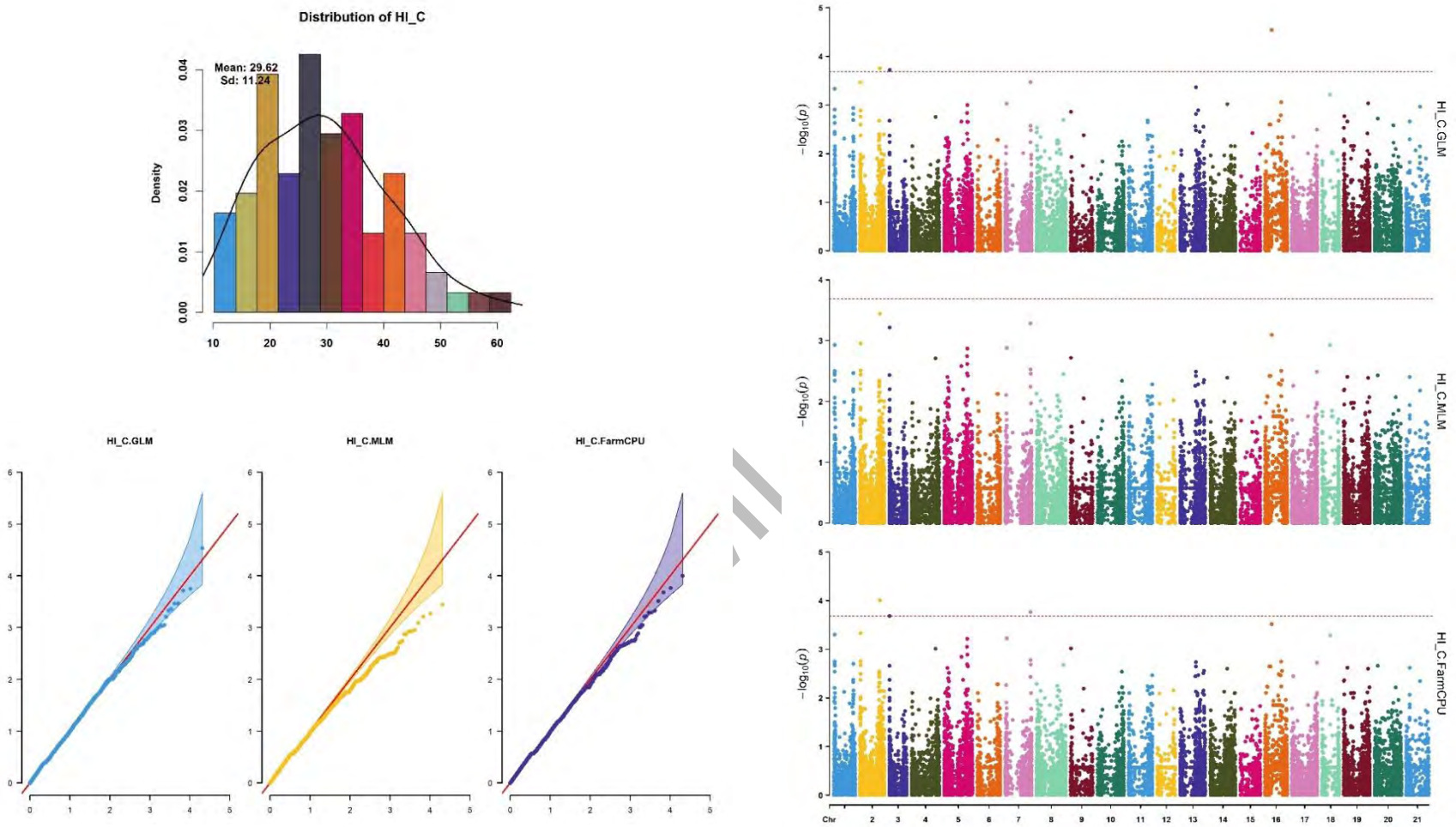
Appendix 2.15. The Density Distribution plot, QQ-plot, and Manhattan plot for grain per spike under control; GpS_C. (a) The density plot is showing the distribution of GpS_C in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



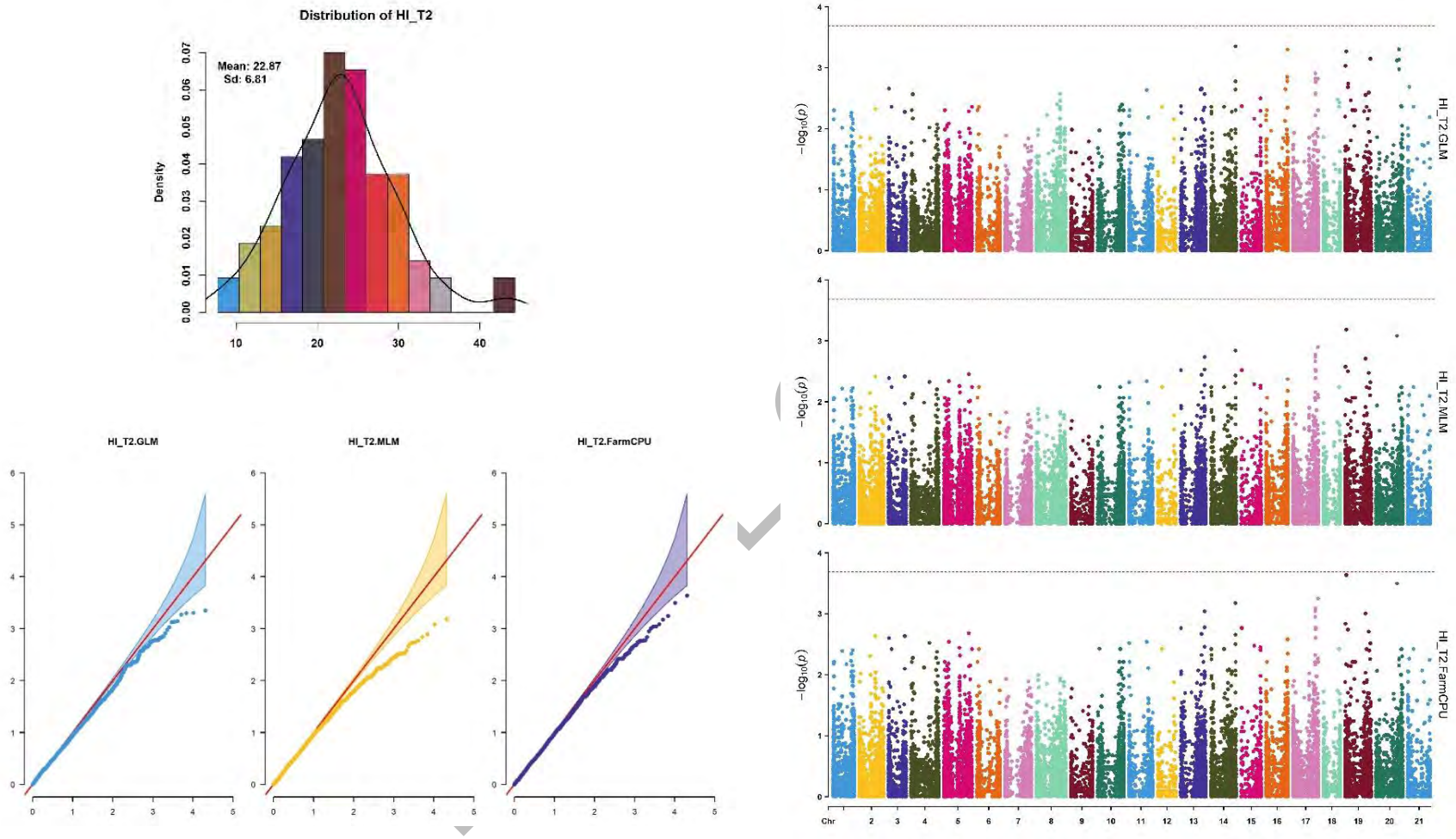
Appendix 2.16. The Density Distribution plot, QQ-plot, and Manhattan plot for grain per spike under treatment 1; GpS_T1. (a) The density plot is showing the distribution of GpS_T1 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



Appendix 2.17. The Density Distribution plot, QQ-plot, and Manhattan plot for grain per spike under treatment 2; GpS_T2. (a) The density plot is showing the distribution of GpS_T2 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



Appendix 2.18. The Density Distribution plot, QQ-plot, and Manhattan plot for harvest index under control; HI_C. (a) The density plot is showing the distribution of HI_C in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis.



Appendix 2.19. The Density Distribution plot, QQ-plot, and Manhattan plot for harvest index under treatment 2; HI_T2. (a) The density plot is showing the distribution of HI_T2 in selected panel, (b) QQ-plot is representing deviation of the obtained p values from the expected values in GLM, MLM, and FarmCPU, and (c) Manhattan plot is showing the P values of the entire GLM, MLM, and FarmCPU analysis

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Appendix 4.1. Agro-physiological traits of twelve wheat varieties affected by variable nitrogen levels

Traits	Plant Height (cm)			Tiller per Plant			Relative SPAD Index			Canopy Temperature Depression (˚C)			Nitrogen Agronomic Efficiency (kg/Kg)			Relative Normalized Difference Vegetation Index		
N Levels	2016-17	2017-18	Mean	2016-17	2017-18	Mean	2016-17	2017-18	Mean	2016-17	2017-18	Mean	2016-17	2017-18	Mean	2016-17	2017-18	Mean
N120	95.22a	97.2a	96.21	4.66a	4.83a	4.75	-	-	-	5.41a	5.47a	5.44	5.79a	5.94a	5.87	-	-	-
N60	94.33b	94.16b	94.25	4.16b	4.38b	4.28	0.84a	0.88a	0.88	4.65b	5.07b	4.86	3.82b	3.93b	3.88	0.96a	0.94a	0.96
N0	91.71c	92.07c	91.90	3.88b	4.16b	4.03	0.71b	0.78b	0.75	3.325c	3.57c	3.45	-	-	-	0.74b	0.75b	0.75
Varieties																		
FSD-08	116.41a	108.42b	112.42	6.22a	6.44a	6.33	0.99a	0.99a	0.99	5.22a	5.31a	5.27	0.94j	3.38g	2.16	1.02a	1.03a	1.03
PIRSBK-08	109.71b	102.28c	106.00	5.55ab	5.88ab	5.72	0.97a	0.96ab	0.97	5.06b	5.08bc	5.08	2.58h	5.21e	3.90	1.01b	1.01ab	1.00
NARC-09	107.76c	100.92c	104.34	5.33ab	5.33bc	5.33	0.91bc	0.95b	0.94	4.97b	5.22ab	5.10	2.14i	6.23d	4.18	0.95c	1.01ab	0.98
TD-1	102.28e	96.23e	99.26	4.66bcd	4.77cd	4.72	0.91bc	0.92c	0.92	4.81c	5.13bc	4.97	4.34f	5.67e	5.01	0.93d	0.96c	0.95
T-8	105.81d	110.16a	107.99	4.77bc	5c	4.89	0.92b	0.93bc	0.93	4.98b	5.02cd	5.01	2.13i	8.48b	5.31	0.95c	0.98bc	0.97
AAS-11	91.68g	92.57f	92.13	3.77def	4.11def	3.94	0.82d	0.87d	0.85	4.66d	4.83ef	4.75	5.63d	1.53i	3.58	0.89e	0.89d	0.90
PAKISTAN-13	75.07j	96.9de	85.99	4.33cde	4.67cde	4.50	0.89c	0.89d	0.89	4.75cd	4.94de	4.85	9.02a	3.98f	6.50	0.91d	0.92d	0.92
CHAKWAL-50	72.92k	86.06g	79.49	3.55ef	3.89efg	3.72	0.79e	0.82e	0.81	4.41e	4.78fg	4.60	4.26g	2.07h	3.17	0.85f	0.85e	0.85
GA-2002	83.09h	76.51j	79.80	3.55ef	3.78fg	3.67	0.77e	0.77f	0.77	4.21f	4.67g	4.44	5.16e	4.22f	4.69	0.81g	0.72f	0.76
INQILAB-91	80.37i	83.98h	82.18	3.33fg	3.67fg	3.50	0.61f	0.71g	0.66	3.78g	4.21h	3.99	7.36c	9.35a	8.36	0.73h	0.61h	0.67
SH-2002	83.76h	98.62d	91.19	3.22fg	3.22gh	3.22	0.49g	0.61h	0.56	3.41h	3.84i	3.63	8.46b	7.43c	7.95	0.66i	0.64g	0.65
AARI-11	96.23f	81.14i	88.69	2.55g	2.78h	2.67	0.45h	0.55i	0.50	3.21i	3.41j	3.31	5.65d	5.68hi	5.66	0.56j	0.56i	0.56
ANOVA values																		
N-levels	***	***		**	**		***	***		***	***		***	***		***	***	
Varieties	***	***		***	***		***	***		***	***		***	***		***	***	
N-levels* Varieties	***	***		NS	**		***	***		***	***		***	***		***	***	

*Significant at $p \leq 0.05$, **Significant at $p \leq 0.01$, ***Significant at $p \leq 0.001$, NS non-significant, Mean values of different N-levels and wheat varieties having different alphabetical letters are different from each other with significant variation

Appendix 4.2. Yield and yield related traits of twelve wheat varieties affected by variable nitrogen levels

Traits	Grains per Spike			Spike Length (cm)			Thousand Kernel weight (TKW)			Biological Yield (kg/ha)			Grain Yield (kg/ha)			Harvest Index (%)		
N Levels	2016-17	2017-18	Mean	2016-17	2017-18	Mean	2016-17	2017-18	Mean	2016-17	2017-18	Mean	2016-17	2017-18	Mean	2016-17	2017-18	Mean
N120	52.08a	52.81a	52.44	9.46a	9.856a	9.66	44.33a	52.81a	48.57	11015a	11117a	11066.00	3116.6a	3151.7a	3134.15	28.06a	28.12a	28.10
N60	48b	48.80b	48.40	8.68b	9.072b	8.88	40.63b	48.81b	44.72	10499b	10711b	10605.00	2650.5b	2675b	2662.75	24.52b	24.26b	24.39
N0	43.27c	44.11c	43.69	8.28c	8.66c	8.48	38.36c	44.11c	41.24	10024c	10202c	10113.00	2421.2c	2438.9c	2430.05	23.27c	22.99c	23.13
Varieties																		
FSD-08	64.11a	65.22a	64.67	11.23a	11.78a	11.51	48.34a	48.87a	48.61	12564a	13096a	12830.00	3747.3a	3890.9a	3819.10	29.84d	29.71c	29.78
PIRSBK-08	63.33a	63.77a	63.56	10.74a	10.93ab	10.84	47.78a	48.5a	48.14	12391b	12813b	12602.00	3696.7b	3691.1b	3693.90	29.71e	28.81e	29.26
NARC-09	53.77b	54.44b	54.11	9.78b	10.17bc	9.98	47.54a	48.01ab	47.78	12127c	12463c	12295.00	3681.2c	3654.6c	3667.90	30.47b	29.33d	29.90
TD-1	47.88d	48.11d	48.00	9.37bc	9.72cde	9.54	43.06c	43.28c	43.18	11945d	11334e	11639.50	3598.6d	3612.1d	3605.35	30.11c	31.86a	30.98
T-8	50.33c	51c	50.67	9.31bc	9.91cd	9.61	45.36b	46.28b	45.83	11022e	11500d	11261.00	3456e	3479.1e	3467.55	31.35a	30.25b	30.81
AAS-11	43.77e	45.22ef	44.50	8.72de	9.11def	8.92	40.54d	41.25c	40.90	10772f	10868f	10820.00	2075.7i	2147.2h	2111.45	19.15j	19.61i	19.38
PAKISTAN-13	44.44e	46.78de	45.61	9.11cd	9.54cde	9.33	42.13c	42.38c	42.26	10507g	10911f	10709.00	3033.8f	3066f	3049.90	28.75f	28.01f	28.38
CHAKWAL-50	43.33e	44.22fg	43.78	8.43e	8.98ef	8.71	39.97d	42.05c	41.02	10379h	10513g	10446.00	2124.9h	2142.2h	2133.55	20.44h	20.33h	20.39
GA-2002	42.77ef	43.56g	43.17	7.87f	8.38fg	8.12	37.92e	38.81d	38.37	9482i	9495h	9488.50	2553.6g	2564.3g	2558.95	26.75g	26.83g	26.79
INQILAB-91	41fg	41.78h	41.39	7.7f	8.02gh	7.86	34.68f	35.24e	34.97	8821j	8892i	8856.50	1642.4k	1655j	1648.70	18.41l	18.61j	18.51
SH-2002	40.11gh	40.89h	40.50	7.11g	7.32hi	7.22	33.42fg	34.08e	33.76	8765k	8845i	8805.00	1748.8j	1761i	1754.90	19.94i	19.65i	19.80
AARI-11	38.56h	37.88i	38.22	6.34h	6.48i	6.41	32.53g	33.2e	32.87	7378l	7389j	7383.50	1394.1l	1398.7k	1396.40	18.48k	18.52j	18.51
ANOVA values																		
N-levels	***	***		***	***		***	***		***	***		***	***		***	***	
Varieties	***	***		***	***		***	***		***	***		***	***		***	***	
N-levels* Varieties	***	***		***	***		***	**		***	***		***	***		***	***	

*Significant at p≤0.05, **Significant at p≤0.01, ***Significant at p≤0.001, NS non-significant, Mean values of different N-levels and wheat varieties having different alphabetical letters are different from each other with significant variations

Appendix 4. 3. Multiple Linear regression to show the relationship of RSI and RNDVI with agro-physiological traits of 12 wheat varieties grown under three N-levels

Trai	Source	DF	R^2 Lost If Term(s) Removed	Sum of Squares	Mean Square	F-Ratio	Prob Level
GY	Intercept	1		1.556175E+08	1.556175E+08		
	Model	2	0.7507	1.715061E+07	8575306	31.624	0.0000
	RSI	1	0.2651	6055497	6055497	22.331	0.0001
	RNDVI	1	0.0242	552153.9	552153.9	2.036	0.1683
	Error	21	0.2493	5694487	271166		
	Total	23	2.28451E+07	993265.2			
BY	Intercept	1		2.575423E+09	2.575423E+09		
	Model	2	0.7925	5.847634E+07	2.923817E+07	40.106	0.0000
	RSI	1	0.2296	1.694473E+07	1.694473E+07	23.243	0.0001
	RNDVI	1	0.0100	737667.1	737667.1	1.012	0.3259
	Error	21	0.2075	1.530963E+07	729029.9		
	Total	23		7.378597E+07	3208086		
PH	Intercept	1		207904.7	207904.7		
	Model	2	0.3925	1362.862	681.4308	6.785	0.0053
	RSI	1	0.0850	294.9657	294.9657	2.937	0.1013
	RNDVI	1	0.0003	1.06936	1.06936	0.011	0.9188
	Error	21	0.6075	2109.175	00.4369		
	Total	23		3472.037	150.9581		
HI	Intercept	1		13551.72	13551.72		
	Model	2	20.6665	596.0598	298.0299	20.982	0.0000
	RSI	1	0.2514	224.8367	224.8367	15.829	0.0007
	RNDVI	1	0.0277	24.81087	24.81087	1.747	0.2005
	Error	21	0.3335	298.2841	14.20401		
	Total	23		894.3439	38.88452		
GpS	Intercept	1		50891.39	50891.39		
	Model	2	0.6017	1143.74	571.8701	15.864	0.0001
	RSI	1	0.1566	297.5691	297.5691	8.255	0.0091
	RNDVI	1	0.0038	7.194865	7.194865	0.200	0.6596
	Error	21	0.3983	757.0087	36.04803		
	Total	23		1900.749	82.64125		
SL	Intercept	1		1806.424	1806.424		
	Model	2	0.7740	35.10875	17.55438	35.959	0.0000
	RSI	1	0.2775	12.58584	12.58584	25.781	0.0000
	RNDVI	1	0.0265	1.201305	1.201305	2.461	0.1317
	Error	21	0.2260	10.25179	0.4881806		
	Total	23		45.36054	1.972198		
T/P	Intercept	1		413.8935	413.8935		
	Model	2	0.6623	18.68943	9.344714	20.596	0.0000
	RSI	1	0.2362	6.664959	6.664959	14.690	0.0010
	RNDVI	1	0.0222	0.6263348	0.6263348	1.380	0.2532
	Error	21	0.3377	9.528165	0.4537221		
	Total	23		28.21759	1.226852		
	Intercept	1		38085.98	38085.98		
	Model	2	0.8377	729.2223	364.6111	54.190	0.0000

TKW	RSI	1	0.2336	203.3682	203.3682	30.225	0.0000
	RNDVI	1	0.0084	7.3384	7.3384	1.091	0.3082
	Error	21	0.1623	141.2959	6.728374		
	Total	23		870.5181	37.84861		
CTD	Intercept	1		434.7759	434.7759		
	Model	2	0.6847	17.49963	8.749816	22.800	0.0000
	RSI	1	0.1258	3.215242	3.215242	8.378	0. 0087
	RNDVI	1	0.0001	0.00290437	0.00290437	0.008	0.9315
	Error	21	0.3153	8.059153	0.3837692		
	Total	23		25.55878	1.111251		

*Plant height (PH), tillers per plant (TpP), canopy temperature as canopy temperature depression (CTD), grains per spike (GpS), spike length (SL), thousand kernel weight (TKW), biological yield (BY), grain yield (GY) and harvest index (HI

QUAID-I-AZAM UNIVERSITY, ISLAMABAD
DEPARTMENT OF PLANT SCIENCES

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It is certified that **Ms. Tayyaba Andleeb**, Registration No. **03041613005,** has published two research articles entitled as "Wheat Varietal Response to Relative SPAD Index (RSI) and Relative Normalized Difference Vegetation Index (RNDVI) under Variable Nitrogen Application and Terminal Heat Stress along with Yield Repercussion" in **"Agronomy"** and "Wheat NAM genes regulate the majority of early monocarpic senescence transcriptional changes including nitrogen remobilisation genes" in **"G3: Genes|Genomes|Genetics"; W-Category Journals** having an **impact factor 3.417** and **3.154** from her dissertation entitled as "Omics Approaches to Decipher Nitrogen Response in Bread Wheat".

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Department of Plant Sciences
Quaid-i-Azam University
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Wheat Varietal Response to Relative SPAD Index (RSI) and Relative Normalized Difference Vegetation Index (RNDVI) under Variable Nitrogen Application and Terminal Heat Stress along with Yield Repercussion

Tayyaba Andleeb [1,*], Zeshan Ali [2], Zahid Mahmood [3], Sadia Latif [1,4] and Umar Masood Quraishi [1,*]

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[1] Department of Plant Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad 45320, Pakistan; slatif@bs.qau.edu.pk
[2] Plant Physiology Program, Crop Sciences Institute, National Agricultural Research Centre, Park Road, Islamabad 45500, Pakistan; eco4nd@yahoo.com
[3] Wheat Programme, National Agricultural Research Centre, Islamabad 44000, Pakistan; zeearid@gmail.com
[4] Department of Biology, Faculty of Sciences, Allama Iqbal Open University, Islamabad 44040, Pakistan
*Correspondence: tayyabanawz@gmail.com (T.A.); umasood@qau.edu.pk (U.M.Q.)

Abstract: Nitrogen (N) deficiency and heat stress (HS) are major abiotic stresses that affect the quantity and quality of wheat grains. This study was conducted to examine wheat varietal response to RSI and RNDVI at the anthesis stage and their relationship to yield and yield-related traits under variable N supply and terminal heat stress. Twelve wheat varieties were evaluated in 2016–2017 and 2017–2018 at the National Agricultural Research Centre (NARC), Islamabad, Pakistan. The experiment was divided into three sets, i.e., N120 (120 kg N/ha), N60 (60 kg N/ha) and N0 (0 kg N/ha), based on the nitrogen fertilizer application. The physiological and yield-related parameters were recorded. Mean grain yield for all twelve varieties, averaged from two years of data, ranged between 1655.0 and 3890.1 kg/ha. Maximum RSI (0.99), RNDVI (1.03) and GY (3890.9 kg/ha) were recorded for FSD-08, while AARI-11 showed minimum RSI (0.50), RNDVI (0.56) and GY (1396.40 kg/ha). In the present study, mean CTD was lower, at N0 (3.57 ° C), followed by N60 (5.07 ° C) and N120 (5.47 ° C) on average for the two years of data. The strong positive correlation of RSI and RNDVI with grain yield at $R^2 = 0.73$ and $R^2 = 0.49$ suggest that these parameters can be used as efficient and precise selection criteria for identifying nitrogen-use-efficient wheat varieties under terminal heat-stress conditions. This work will help the researchers to identify and develop nitrogen-use efficient and thermos-tolerant wheat cultivars by minimizing the negative impacts of heat stress at the anthesis stage.

Keywords: canopy temperature; heat stress; nitrogen; varieties; wheat; yield

Introduction

Wheat crop covers 17% of the world crop cultivated area and contributes to approximately 20% of the total calories in the human diet [1]. It is a staple cereal crop for 40% of the world population [2]. Major constraints for wheat production are abiotic stresses, including low soil fertility, nutrient deficiency, heavy metal stress, moisture deficit, salinity stress, drought stress and heat stress [3]. Heat stress is one of major challenges that significantly impacts wheat yield, and it occurs repeatedly during the cropping season [4]. In current climatic conditions, rising temperatures are a serious threat that can cause tremendous decreases in wheat production [5]. It reduces crop yield through alterations in physiological processes, such as photosynthesis, protein denaturation, increased amount of fatty acids accumulation, membrane thermos-stability, and starch synthesis. It also accelerates vegetative growth, ultimately leading to decreased grain filling duration [6,7]. One important strategy to overcome losses due to heat stress is the selection of heat-tolerant genotypes that could be better adapted to high temperature, thus maintaining the desired yield [8]. Besides this breeding approach, wheat yield under heat stress could be maintained and improved through modified crop micro-climatic conditions.

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Plant Genetics and Genomics

Wheat NAM genes regulate the majority of early monocarpic senescence transcriptional changes including nitrogen remobilization genes

Tayyaba Andleeb [1,2], Emilie Knight [1], Philippa Borrill [1,*]

[1]Department of Crop Genetics, John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK,
[2]Department of Plant Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad 15320, Pakistan

*Corresponding author: Department of Crop Genetics, John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK. Email: philippa.borrill@jic.ac.uk

Abstract

Senescence enables the remobilization of nitrogen and micronutrients from vegetative tissues of wheat (Triticum aestivum L.) into the grain. Understanding the molecular players in this process will enable the breeding of wheat lines with tailored grain nutrient content. The NAC transcription factor NAM-B1 is associated with earlier senescence and higher levels of grain protein, iron, and zinc contents due to increased nutrient remobilization. To investigate how related NAM genes control nitrogen remobilization at the molecular level, we carried out a comparative transcriptomic study using flag leaves at 7 time points (3, 7, 10, 13, 15, 19, and 26days after anthesis) in wild type and NAM RNA interference lines with reduced NAM gene expression. Approximately 2.5 times more genes were differentially expressed in wild type than NAM RNA interference plants during this early senescence time course (6,508 vs 2,605 genes). In both genotypes, differentially expressed genes were enriched for gene ontology terms related to photosynthesis, hormones, amino acid transport, and nitrogen metabolism. However, nitrogen metabolism genes including glutamine synthetase (GS1 and GS2), glutamate decarboxylase (GAD), glutamate dehydrogenase (GDH), and asparagine synthetase (ASN1) showed stronger or earlier differential expression in wild-type than in NAM RNA interference plants, consistent with higher nitrogen remobilization. The use of time course data identified the dynamics of NAM-regulated and NAM-independent gene expression changes during senescence and provides an entry point to functionally characterize the pathways regulating senescence and nutrient remobilization in wheat.

Keywords: Triticum aestivum L. (wheat); senescence; transcription factors; nitrogen remobilization; flag leaf; NAM-B1; Gpc-B1; Plant Genetics and Genomics

Introduction

Wheat supplies approximately 20% of calories in the human diet and is an important source of protein and micronutrients. Beyond nutritional benefits, wheat grains with higher protein content are associated with increased breadmaking quality and attract a price premium. Although nitrogen (N) fertilization is commonly used to increase grain protein content, high nitrogen fertilization leads to higher production costs and environmental pollution (Aranguren et al. 2021; Martınez-Dalmau et al. 2021). Alternatively, genetic approaches can be used to increase protein content, although identifying the genetic loci to target remains a challenge.

The final grain yield and nutrient content depends on the accumulation and transport of carbon, nitrogen and other nutrients from the vegetative tissues to the developing grain. The remobilization of nutrients is strongly influenced by the process of senescence, which is a developmentally regulated programme to remobilize nutrients from vegetative tissues to the developing grain. The starting time and progression of flag leaf senescence influences the remobilization of nutrients and the final yield (Distelfeld et al. 2014), with the flag leaf contributing a significant proportion of nitrogen to the seed by degrading and recycling proteins (Kichey et al. 2007; Bogard et al. 2010; Have´ et al. 2017). Delayed leaf senescence can be associated with prolonged photosynthesis and increased grain yield but also decrease grain protein content due to reduced nutrient remobilization from the leaf tissues (Uauy et al. 2006; Alpuerto et al. 2021). Therefore, altering the rate and progress of senescence can influence final yield and protein content of wheat grain. Understanding the molecular components influencing flag leaf senescence and nitrogen remobilization can help to improve nitrogen remobilization efficiency and grain protein content in wheat. The identification of the NAM-B1 gene which is a NAC transcription factor that influences senescence and grain nutrient content opens the door to identify the molecular pathways regulating senescence and nutrient remobilization in wheat. NAM-B1 was identified through positional cloning as the causal gene for Gpc-B1 which affects grain protein content (Uauy et al. 2006). NAM-B1, together with its homoeologs NAM-A1 and NAM-D1, influences senescence and enhances nutrient remobilization (Avni et al. 2014; Cormier et al. 2015; Harrington et al. 2019). Most modern wheat cultivars carry a nonfunctional allele of NAM-B1, whereas the functional allele, which was identified through mapbased cloning, is mainly found in wild emmer.