IDENTIFICATION OF SNP MARKERS ASSOCIATED WITH RESISTANCE TO *PYRENOPHORA TERES* F. *TERES* ON BARLEY

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ÜLKÜ SELCEN HAYDAROĞLU

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

JANUARY 2024

Approval of the thesis:

IDENTIFICATION OF SNP MARKERS ASSOCIATED WITH RESISTANCE TO *PYRENOPHORA TERES* F. *TERES* ON BARLEY

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ABSTRACT

IDENTIFICATION OF SNP MARKERS ASSOCIATED WITH RESISTANCE TO *PYRENOPHORA TERES* F. *TERES* ON BARLEY

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January 2024, 143 pages

Pyrenophora teres f. *teres* (*Ptt*) is the causal fungal agent of barley net-type net-blotch (NTNB) disease which can cause significant yield losses. In this study, phenotyping and genotyping data were obtained from a biparental barley doubled haploid (DH) mapping population of 277 DH lines inoculated with a virulent *Ptt* isolate (GPS18). The DH population was derived from the second-generation hybrids (F_2) of a disease-resistant (Avcı 2002, "A") and a susceptible (Bülbül 89, "B") variety using anther culture technique. The pretreated anthers in 1.0 M mannitol showed statistically a better response than 0.7 M mannitol for A × B F_2 hybrids. The callus induction ratio was significantly the highest at 37.6% for the "induction" medium, while the ratio of green plant formation was statistically the highest at 24.7% for the "R9" medium. Sequencing-based diversity array technology (DArT-seq) allowed the identification of 9,170 SNP markers, providing the construction of a linkage map of 1682.97 cM length, with an average density of the markers in 1.49 marker/cM. This led to the identification of three quantitative trait loci (QTLs) for *Ptt* on 3H, 4H, and 6H barley chromosomes. The QTL on the 6H was likely

overlapping the previously reported *SFNB-6H-33.74* locus, while the QTL on the 3H was potentially novel. On the other hand, the QTL on the 4H might be covering the *Rpt7* locus. The percentage of phenotypic variation explained (PVE) by these QTLs was almost 26%, cumulatively. Disease-resistance-associated SNPs identified within these QTLs can be used for developing DNA tests.

Keywords: *Pyrenophora teres* f. *teres*, Genetic mapping, DArT-seq, QTL, Barley.

ÖZ

ARPADA *PYRENOPHORA TERES* F. *TERES* ETMENİNE DAYANIKLILIKLA İLİŞKİLİ SNP MARKÖRLERİNİN TANIMLANMASI

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Ocak 2024, 143 sayfa

Pyrenophora teres f. *teres* (*Ptt*), arpada ağ-tipi ağ benek (NTNB) hastalığına neden olan fungal bir etmen olup; önemli oranda verim kayıplarına sebep olabilmektedir. Bu çalışmada, virülent bir *Ptt* izolatı (GPS18) ile inokule edilmiş, 277 double haploid (DH) bireyden oluşan iki ebeveynli arpa DH haritalama popülasyonundan fenotipleme ve genotipleme verileri elde edilmiştir. DH popülasyonu, hastalığa dayanıklı (Avcı 2002, "A") ve hassas (Bülbül 89, "B") çeşitlerin ikinci nesil melezlerinden (F_2), anter kültür tekniği kullanılarak geliştirilmiştir. Analizler sonucunda, A × B F_2 hibritlerinden izole edilen 1.0 M mannitol ile ön muamele edilmiş anterler, 0.7 M mannitole kıyasla istatistiksel olarak daha iyi yanıt göstermiştir. Kallus oluşum oranı, "indüksiyon" ortamı için %37,6 ile önemli derecede en yüksek orana sahip olarak bulunmuş olup; yeşil bitki oluşum oranı ise "R9" ortamı için %24,7 ile istatistiksel olarak anlamlı en yüksek değeri almıştır. Sekanslama tabanlı çeşitlilik dizileri teknolojisi (DArT-seq), 9170 SNP markörünün tanımlanmasına izin vererek; ortalama 1,49 markör/cM yoğunluk ile 1682,97 cM uzunluğunda bir bağlantı haritasının oluşturulmasını sağlamıştır. Böylece, 3H, 4H ve 6H arpa kromozomlarında *Ptt* için üç nicel özellik lokusu (QTL) tanımlanmıştır.

Bulunan QTL’lerden 6H üzerindeki QTL, daha önce bildirilen *SFNB-6H-33.74* lokusu ile muhtemelen örtüşmekte olup; 3H üzerindeki QTL potansiyel olarak yeni olabilir. Öte yandan, 4H üzerindeki QTL’in *Rpt7* lokusunu kapsıyor olabileceği görülmüştür. Toplamda bu QTL'lerin açıkladığı fenotipik varyasyon yüzdesi (PVE) yaklaşık %26 olarak bulunmuştur. Tanımlanan QTL’lerdeki hastalığa dayanıklılıkla ilişkili SNP'ler, DNA testlerinin geliştirilmesinde kullanılabilecektir.

Anahtar Kelimeler: *Pyrenophora teres* f. *teres*, Bağlantı haritalaması, DArT-seq, QTL, Arpa.

To my family and my husband,

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my supervisor, Assist. Prof. Dr. Aybar Can Acar, and co-supervisor, Prof. Dr. Aziz Karakaya, for their invaluable guidance, advice, and support throughout my thesis journey. Special thanks to Assoc. Prof. Dr. Arzu Çelik Oğuz for her insightful contributions to disease testing methods. My sincere appreciation goes to my thesis examining committee, Prof. Dr. Zeki Kaya, Prof. Dr. Emre Keskin, Assist. Prof. Dr. Güray Akdoğan and Assist. Prof. Dr. Emre Aksoy for their constructive criticism and direction. I am grateful to the Republic of Turkey, The Ministry of Agriculture and Forestry, General Directorate of Agricultural Research and Policies (TAGEM), for their financial support through the TAGEM project (under grant number TAGEM/TBAD/B/19/A7/P1/1259). I also thank the Field Crops Central Research Institute for the financial contributions to the sequencing analyses. Heartfelt thanks to Dr. Gülden Çetin-Özkan for her mentorship in the doubled haploidy technique, and to my friend, Dr. Shaun Clare, for his invaluable contributions in *in silico* analyses. I extend my thanks to Assist. Prof. Dr. Fatih Ölmez and Dr. Namuk Ergün for their unwavering support during my Ph.D. studies. Also, my appreciation goes to Gizem Demirel, Dr. Sümeyra Gültekin, and Firdevs Özbek who supported me both academically and as friends during a milestone of my life.

I owe a huge debt of gratitude to my beloved father, Nevzat Demir, and my mother, Ayten Demir who made me feel extremely fortunate in my life and never left me alone at any moment. I am indebted to express my gratitude to my sister Gülsüm Gökçen Akbulut, for her unending love, support, and belief in me. Finally, I would like to thank my husband, Ob/Gyn Yasin Haydaroğlu, for the understanding he provided me in completing my Ph. D. journey.

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LIST OF ABBREVIATIONS

ABBREVIATIONS

AFLPs	Amplified Fragment Length Polymorphisms
AM	Association Mapping
Avr	Avirulence
BAM	Binary Version of SAM
BC1	First Backcross
BILs	Backcross Inbred Lines
BWA	Burrows-Wheeler Alignment Tool
CAPS	Cleaved Amplified Polymorphic Sequences
CIM	Composite Interval Mapping
CRoPs	Complexity Reduction of Polymorphic Sequences
DArT	Diversity Array Technology
DdNTP	Dideoxynucleotides
DdRAD	Double-Digest RAD-Seq
DH	Doubled Haploid
ETI	Effector-Triggered Immunity
F_1	First Generation Hybrid
F_2	Second Generation Hybrid
FCCRI	Field Crops Central Research Institute
GBS	Genotyping by Sequencing
GLM	General Linear Model
GWAS	Genome Wide Association Studies
HR	Hypersensitive Response
IBSC	International Barley Sequencing Consortium
IMP	Multiple Imputation
ISSRs	Inter Simple Sequence Repeats
KASP	Kompetitive Allele Specific PCR
LD	Linkage Disequilibrium
LG	Linkage Group
LOD	Logarithm of Odds
MAGIC	Multiparental Advanced Generation Intercrosses
MAMs/PAMPs	Microbe/Pathogen Associated Molecular Patterns
MAS	Marker-Assisted Selection

MIP	Molecular Inversion Probe
MLM	Mixed Linear Model
NAM	Nested Association-Mapping
NB	Net Blotch
NBS-NLR	Nucleotide Binding Site-Leucine Rich Repeats
NEs	Necrotrophic Effectors
NETS	Necrotrophic Effector-Triggered Susceptibility
NGS	Next Generation Sequencing
NILs	Near Isogenic Lines
NTNB	Net Type Net Blotch
PCD	Programmed Cell Death
PRRs	Protein Recognition Receptors
PTI	PAMP-Triggered Immunity
Ptm	*Pyrenophora teres* form *maculata*
Ptt	*Pyrenophora teres* form *teres*
QTL	Quantitative Trait Locus
QTLs	Quantitative Trait Loci
RAD	Restriction-Site Associated DNA
RAPDs	Random Amplification of Polymorphic DNAs
RE	Restriction Enzymes
RFLPs	Restriction Fragment Length Polymorphisms
RILs	Recombinant Inbred Lines
RRL	Reduced Representation Libraries
RT	Room Temperature
SAM	Sequence Alignment Map
SCARs	Sequence Characterized Amplified Region
SdRAD	Single-Digest in RAD-Seq
SIM or IM	Standard Interval Mapping
SMR	Single Marker Regression
SNP	Single Nucleotide Polymorphism
SSR	Simple-Sequence Repeats
STNB	Spot Type Net Blotch
WGS	Whole Genome Resequencing

CHAPTER 1

INTRODUCTION

Sustainable agriculture is important to meet the increasing food demand due to the growing world population, climate change, and corruption of arable areas. The production of high-yielding crops with disease resistance enhanced nutritional quality, and abiotic and biotic stress tolerance traits play a critical role in agricultural sustainability. Sustainable and increased production of the major cereal crops is a critical issue to be taken into consideration.

Barley, belonging to the grass family, has great agronomical importance as one of the major cereal crops worldwide. It is globally the fourth most abundant cereal after wheat, maize, and rice, considering the amount of production and cultivation area (El-Hashash & El-Absy, 2019). It is a nutritious crop containing 78% carbohydrates and 10% proteins (El-Hashash & El-Absy, 2019) with vitamins (especially B vitamins including niacin, thiamin, pyridoxine), dietary minerals, and fibers such as beta-glucans (Baik & Ullrich, 2008; Jan et al., 2022). Currently, the highest consumption share for barley usage is in the feed industries, followed by the malting and brewing industries (Tricase et al., 2018). Even though a small share of barley is consumed as food, the share-out for feeding millions of people cannot be underestimated in terms of global food security.

Barley is the second major crop in Turkey following wheat, both in terms of cultivation and production (Karagöz, 2017). As a cool climate field crop, barley can be planted in almost any region due to its adaptation to a broad spectrum of agroclimatic conditions (Meng et al., 2023). Depending on the abiotic or biotic stress levels that the crops encountered, the barley yield is quite variable each year (Figure 1.1 and Table 1.1). In 2021, barley planting areas in Turkey showed an approximate

increase of 2.3% compared to 2020. The total barley production of Turkey in 2021 was about 5.8 million tons, which is a 30.7% decrease from the previous year. When looking at the yield, in 2021, the yield decreased by approximately 32.5% compared to the previous year, becoming 181 kg/da (Eğilmez, 2022). The dry conditions in Turkey during 2021 have led to a reduction in the production of barley. According to the Turkish Statistical Institute (TÜİK) data, in Turkey, barley was sown into approximately 3 million hectares of area and its production increased by 47.8% compared to the previous year, and reached 8.5 million tonnes in 2022 (www.data.tuik.gov.tr).

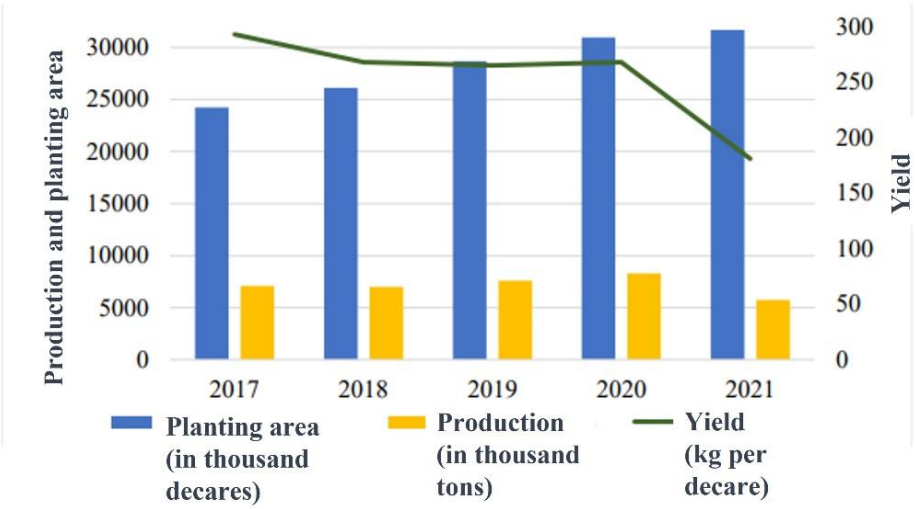

Figure 1.1. Barley planting area, production, and yield in Turkey (Eğilmez, 2022).

Table 1.1 Barley planting area, production, and yield in Turkey (Eğilmez, 2022).

Year	Planting area (Thousand da)	Production (Thousand tons)	Yield (kg/da)
2017	24,247	7,100	293
2018	26,119	7,000	268
2019	28,690	7,600	265
2020	30,972	8,300	268
2021	31,691	5,750	181

One of the major economically important fungal diseases affecting barley is net blotch (NB), an important foliar disease (Ronen et al., 2019). It is caused by the necrotrophic fungus *Pyrenophora teres* with two forms designated as *P. teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*). They are the causal agents of net type of net blotch (NTNB) and spot type of net blotch (STNB), respectively (Smedegård-Petersen, 1971). Not only leaves, but also leaf sheaths, stems and kernels are affected in susceptible plants following infection with NB disease-causing agents (Liu et al., 2011). Incidences of NB disease, occurring under disease-inducing environmental conditions, can cause severe disruption in kernel size. Therefore, in susceptible barley cultivars, yield losses range between 10% to 40% (Liu et al., 2011). Moreover, the whole crop can be lost if the infection builds up in susceptible cultivars, under severe epidemics (Shjerve et al., 2014). Solutions need to be developed to manage the risks, considering the potential economic aspects of the disease.

To take this disease under control, fungicides, or cultural methods such as rotation can be applied (Olita et al., 2023). However, these are neither cost-effective nor long-term solutions. Instead, developing resistant cultivars is the most efficient, cost-effective, and environmentalist option (Kumar et al., 2020). However, disease resistance studies can be challenging. In the trials established for the selection of durable plants, natural or artificial inoculations should be carried out and an acceptable proportion of disease should be ensured in the control plants. Suitable environmental conditions (temperature, humidity, and so on), susceptible hosts, and enough inoculum sources should be maintained for the occurrence of the disease. The fact that these conditions do not occur every year hinders the selection of resistant plants. Although it is possible to perform these studies in controlled climatic conditions using artificial inoculation, the results of these studies do not always coincide with the ones occurring under natural inoculation.

There are studies focused on determining the sources of resistance using molecular approaches (Afanasenko et al., 2022; Dinglasan et al., 2019). To be able to efficiently use disease resistance sources, the knowledge of the genetic basis of pathogen and disease resistance of economically important barley cultivars should be better

understood (Singh et al., 2004). To facilitate the selection of enduring plants for resistance breeding, molecular markers seem to be the most effective and efficient approach (Collard & Mackill, 2008). Once the DNA regions associated with disease resistance were identified, it allowed for the selection of resistant plants with high accuracy regardless of the environmental conditions and the pathogen. Therefore, molecular markers need to be identified for their usage in marker-assisted selection (MAS) applications by barley breeders (Dong & Ronald, 2019).

Before the molecular marker technology, some of the NB resistance-conferring gene resources were elucidated using trisomic analysis (Koladia et al., 2017). However, the advent of molecular markers facilitated the discovery of numerous Quantitative Trait Loci (QTLs) associated with NB resistance. There are three types of molecular markers: first, second, and third-generation markers (Khan, 2015). Markers based on DNA hybridization are called the first generation. The second-generation markers are PCR-based, while sequence-based ones are the third-generation markers (Dhutmal et al., 2018). Genotyping by Sequencing (GBS) is defined as a third-generation molecular marker technology and its usage has rapidly increased in various plants for various breeding purposes. GBS technique can be used for detecting single nucleotide polymorphism (SNP) markers in a mapping population to determine QTLs relevant to disease resistance and be further used in breeding programs.

In barley-cultivated areas, *Ptt* is detected prevalently worldwide (Hamany Djande et al., 2023). Both *Ptt* as a fungal agent and barley as a host are complex to study due to genotype-specific interactions (Clare et al., 2021). There are studies indicating that major genes and minor quantitative loci were responsible for host resistance/susceptibility with dominant, recessive, and incomplete characteristics (Liu et al., 2011). On the other hand, numerous pathotypes of the pathogen exist that can infect barley in a genotype-specific manner (Koladia et al., 2017). Therefore, it is crucial to develop molecular markers that are specific to the resistance sources in the durable genotypes that exist in Turkey. Background on the topics related to the current study are given in the literature review in the following chapter.

1.1 Aim, hypothesis, significance, and novelty of the thesis project

In this study, we aimed to identify single nucleotide polymorphism markers (SNPs) associated with the net form of net blotch disease resistance by generating a doubled haploid (DH) mapping population using anthers from F_2 hybrids of NTNB-resistant and susceptible Turkish barley cultivars, Avcı 2002 and Bülbül 89, respectively. We hypothesized that sequencing-based diversity array technology (DArT-seq) analysis of the DH mapping population would provide identification of SNP markers associated, in a statistically significant manner, with NTNB disease resistance in barley using a virulent isolate of *P. teres* f. *teres*. To do so, a DH mapping population was created using anther culture technique, and genotypes in the developed population were genotypically and phenotypically analyzed. Then, *in silico* analyses were conducted through the obtained data to identify QTLs and SNP markers associated with NTNB resistance. Identification of resistance-associated markers is useful for their employment in MAS. Through identifying NTNB resistance-associated SNP markers, kompetitive allele-specific PCR (KASP) markers can be developed for their validation which can be the next stage of this study. Thus, these markers will hopefully contribute to the barley breeding studies for selecting improved varieties. The DH population obtained within the scope of the study is also a very important resource for further mapping studies infuture breeding programs.

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy of barley

The *Hordeum* genus belongs to the Triticeae tribe of the Poaceae (previously known as Gramineae) family which has been thought to be of monophyletic origin until recently (Laugerotte et al., 2022) (Table 2.1). However, according to recent molecular studies, it was hypothesized that the *Hordeum* is a polyphyletic genus (Jeanty et al., 2023). This genus has both annual self-pollinating species (*Hordeum vulgare* and *Hordeum marinum*) and perennial cross-pollinating species (*Hordeum bulbosum*), which differ in the life cycle as well as reproductive behavior (Waugh et al., 2017).

Table 2.1 Taxonomy of barley (El-Hashash & El-Absy, 2019).

Domain:	Eukaryota
Kingdom:	Planta – Plants
Subkingdom:	Tracheobionta - Vascular plants
Phylum:	Spermatophyta - Seed plants
Subphylum:	Magnoliophyta - Angiospermae - Flowering plants
Class:	Liliopsida – Monocotyledonae
Subclass:	Commelinidae
Order:	Cyperales
Family:	Poaceae – Grass
Subfamily:	Pooideae
Tribe:	Triticeae
Genus:	*Hordeum*
Species:	*Hordeum vulgare*

Two barley subspecies are present. Six-row subspecies (with all spikelets fertile), *Hordeum vulgare* subsp. *vulgare*, also called as *Hordeum hexastichum*, and a two-row subspecies (with only central spikelets fertile) called *Hordeum vulgare* subsp. *distichum* (Jeanty et al., 2023). Inside these two subspecies, barley plants can be categorized according to their vernalization needs. Vernalization is the process of meeting the cold need of a crop by exposing it to low temperatures to coordinate the transition from the vegetative stage to reproductive growth (Benkherbache et al., 2016). Winter and spring barley show different characteristics in terms of their vernalization needs and sowing season (Jeanty et al., 2023). Winter barley needs vernalization to promote the flowering stage, while spring one does not (Yan et al., 2006). Therefore, winter barley is required to be sown in autumn (Cha et al., 2022).

There is also a categorization for barley based on it being awned, awnless, hulled (covered), and hulless (naked). Hulled barley is used for feed and malting, while hulless barley which differs in having envelopes of the caryopses is used for food (Hernandez et al., 2020). Hulless barley is easier to process compared to the hulled one (Narwal et al., 2017). Awnless and smooth or rough awned barley can be used for the feed industry, although awnless barley varieties are preferred as forage. The disadvantage of the awnless varieties is their low yields due to the role of awns in photosynthesis. When the awned varieties are used as forage, they need to be harvested shortly after heading to avoid the mature awns or ensiling needs to be done to soften the awns (Jacob & Pescatore, 2012).

2.2 Genetics of barley

Self-pollinated diploid barley has seven chromosomes with a genome size of ~5.1 Gbp and >80% of repetitive elements, the majority of which are transposable elements (TEs) (Sato, 2020). The International Barley Sequencing Consortium (IBSC) has established the reference genome of barley recently (The International Barley Genome Sequencing Consortium, 2012), which accelerated the genetics-based studies on barley by elucidating the genomic positions of markers and

neighboring sequences to them (Riaz et al., 2021). Since 2012, the development of genomic tools for barley has been accelerated due to the existence of a map-based barley genome reference sequence, as well as advances in nextgeneration sequencing (NGS) techniques (Beier et al., 2017; Giraldo et al., 2019). Thus, barley genotyping data of different populations can be analyzed using a reference-based approach for various traits of interest.

2.3 Domestication, and dissemination of barley

Domestication of barley (*Hordeum vulgare*) is an evolutionary process where humans take a role in selecting agents for converting wild barley (*Hordeum spontaneum*) to its cultivated form (El-Hashash & El-Absy, 2019). Wild barley has brittle and smooth rachis, while cultivated barley has more harvestable seeds (Sato, 2020). Cultivated barley has a shorter and thicker spike, as well as larger grains compared to the wild one (Badr et al., 2000). On the other hand, *H. spontaneum* is two-rowed, while cultivated barley can be two-row or six-row. Moreover, outbreeding in *H. spontaneum* appears to be more frequent than in the cultivated form due to its more open-flowered characteristics (Von Bothmer et al., 2003).

Barley domestication has likely multiregional origin and, thus is assumed to have had various independent domestication patterns (Jeanty et al., 2023; Von Bothmer et al., 2003). Agricultural domestication of barley began nearly 11000 years ago in the "Fertile Crescent", where cereals were most likely the first cultivated, with other probable places in Central Asia and Africa (Hernandez et al., 2020; Langridge, 2018). Then, the dissemination of barley possibly occurred throughout Europe and Asia (Hernandez et al., 2020). Elite cultivars that are pure lines, hybrids, or clones were developed approximately 100 years ago, showing better yield, quality, and resistance traits (Bellucci et al., 2013; Sreenivasulu et al., 2008).

2.4 Taxonomy of *Pyrenophora teres* and symptoms of net blotch

Pyrenophora teres belongs to the Fungi kingdom and Ascomycota phylum; while its subphylum is Pezizomycotina, and its class is Dothideomycetes (Backes et al., 2021) (Table 2.2). *Pyrenophora* genus has 135 species. There are two forms of *P. teres*, called *P. teres* f. *teres* and *P. teres* f. *maculata*. *P. graminea*, one of the *Pyrenophora* species, looks like *P. teres* morphologically, but its symptoms (necrotic stripes on leaves) are different from those of the *Ptt* and *Ptm*. Although *P. graminea* and *P. teres* can be crossed due to their close genetic structure, *Ptm* was found as more related to *P. graminea* than *Ptt* (Liu et al., 2011).

Table 2.2 Taxonomy of *P. teres* (Liu et al., 2011).

Domain:	Eukaryota
Kingdom:	Fungi
Subkingdom:	Dikarya
Phylum:	Ascomycota
Subphylum:	Pezizomycotina
Class:	Dothideomycetes
Subclass:	Pleosporomycetidae
Order:	Pleosporales
Family:	Pleosporaceae
Genus:	*Pyrenophora*
Species:	*P. teres*

NTNB symptoms start as pinpoint brown lesions, then turn into dark brown, narrow, longitudinal, and transverse necrotic striations across the leaf blades, which form a net-like pattern. On the other hand, symptoms of STNB are small dark-brown oval spots with chlorosis or necrotic halos (Backes et al., 2021). Although common symptoms are seen on leaves, *Ptm* also infects leaf sheaths (Jordan, 1981).

2.5 Life cycle of *Pyrenophora teres*

The lifestyle of the host determines the characterization of the pathogen as a biotroph (needs living cells of the host), necrotroph (killing the living cells of the host to feed on), or hemibiotroph (first feed on living host cells before switching to necrotrophy) (Kumari et al., 2023). *P. teres* is a haploid hemibiotrophic fungal pathogen that is placed in the heterothallic ascomycetes group. Thus, *P. teres* performs dual infecting phases switching from biotrophy to necrotrophy, while it needs two opposite mating types for sexual recombination as a heterothallic fungus (Martin et al., 2020).

P. teres reproduces through both sexual and asexual stages, although the sexual stage results in higher genetic variability of the pathogen (Backes et al., 2021). Sexual production is a major source of primary infections when two mating types exist in equal frequencies. On the other hand, asexual production tends to occur in case there are unequal frequencies of these mating types (Çelik Oğuz et al., 2018). The sexual stage of the fungus causing NB disease is called *Pyrenophora teres* (Died.) Drechs.; while asexual stage as *Drechslera teres* (Sacc.) Shoem. (Dreschler, 1923; Shoemaker, 1959).

The sexual stage includes fertile ascocarp formation, released from pseudothecia, enabling surviving of the pathogen. Ascospores maintain the dispersal of the pathogen via air or water (Dahanayaka et al., 2021a). On the other hand, conidia (spores) are produced during the asexual reproduction stage. Ascospores and conidia serve as primary inoculum which disseminate the pathogen in short distances. In addition, conidia may serve as the major source of secondary inoculum to facilitate disease spread within the canopy (Liu et al., 2011). *Pyrenophora teres* is capable of surviving in plant debris over seasons and moving into new barley fields with the help of seed-borne mycelium in the embryo and pseudothecia forming on stubble with crop residues (Dahanayaka et al., 2021a; Martin et al., 2018). In a study conducted in Ankara, Turkey conditions, conidia, conidiophores, and pseudothecia were found on the leaves left on the ground and buried. They were more common on the leaves left on the ground. No ascospores were detected in this study. Pycnidia

were found in cooled incubator studies. Fungal cultures survived 0°C and -10°C in a cooled incubator and fungus in diseased leaves survived -10°C. This study showed that fungus can survive during the winter months in Ankara and conidia may have an important role in the infections (Karakaya et al., 2004).

2.6 Genetic structure, virulence diversity and hybridization between forms of *Pyrenophora teres*

The genetic structure of *P. teres* is highly diverse. The sexual production stage of the pathogen results in a novel pathotype resulting in a shift in the genetic structure of the pathogen. This stage is governed by a single mating type locus called *MAT1* with its two alternative forms, *MAT1-1* and *MAT1-2*. Mating between alternative genotypes (*MAT1-1* and *MAT1-2* genotypes) results in the occurrence of ascomata (Dahanayaka et al., 2022).

Virulence can be defined as the pathogen damage level on the host (Koladia et al., 2017). *P. teres* shows high pathogenic variation or virulence diversity for both forms of the pathogen, which results in a challenge for resistance breeding programs. Variations in virulence are dependent on geographic regions and selection pressure on the pathogen (Liu et al., 2011). Since effectors can be host-specific, the virulence of each pathogen isolate can differ for each barley cultivar. Therefore, genomic regions associated with the virulence of the pathogen can be different for each cultivar (Martin et al., 2020). In this respect, the more genomic regions associated with virulence can be identified in barley varieties, the better it is to comprehend the pathosystem.

Hybridization is rare for fungi nature due to reduced fitness and genetic incompatibilities. However, it may take place through sexual production and asexual hyphae fusion (Dahanayaka et al., 2021b). Hybrid forms can result in the emergence of novel virulent pathogens affecting the resistant genotypes currently used in barley breeding (Mironenko et al., 2022). *Ptt* and *Ptm* are phenotypically similar, but they

are genetically different (Alhashel et al., 2021). Laboratory-induced hybrids between these two can be obtained successfully by overcoming fertility barriers. However, sexual production between the two forms cannot happen or is difficult to detect in nature (Dahanayaka et al., 2021b). In a recent study, 5 QTLs conferring virulence and 4 QTLs for leaf symptoms were detected by developing a *Ptt*/*Ptm* hybrid population (Dahanayaka et al., 2022).

2.7 Plant-microbe interactions

Qualitative traits are governed by major gene(s), while quantitative traits are by multiple genes with minor effects or minor and major genes in combination (Merrick et al., 2021). Resistance is a genetically complex trait and can be controlled both qualitatively and quantitatively (St. Clair, 2010). There are two hypotheses about the genetic interaction models of the plants called gene-for-gene interaction and inverse gene-for-gene interaction (Peters Haugrud et al., 2019). According to the gene-for-gene hypothesis, for every resistance (*R*) gene in the host plant, the pathogen also has an avirulence (*Avr*) gene (Grewal et al., 2008). The inverse gene-for-gene interaction hypothesis explains that infection occurs when gene products of the pathogen are recognized by host receptors. Namely, resistance is gained by the host in case there are no receptors for targets of the pathogen (Ayliffe et al., 2022; Fenton et al., 2009).

There are two types of resistance in the plants, called horizontal (non-specific or basal resistance), and vertical resistance (specific resistance). Horizontal resistance is the partial resistance to all pathogen strains. On the other hand, vertical resistance may provide complete resistance to some strains of a pathogen, while not for others (Walton, 1997).

The host has receptors to recognize biotrophic pathogens and activate immune response through programmed cell death (PCD) to stop colonization of the pathogen which needs living tissue of the host. Protein recognition receptors (PRRs) of the

host bind to microbe/pathogen-associated molecular patterns (MAMs/PAMPs) to trigger a plant immune response called PAMP-Triggered Immunity (PTI) (horizontal or basal resistance) (Zhang & Zhou, 2010). This is a typical gene-for-gene interaction.

Effectors released from pathogens govern virulent (compatible or susceptible host) and avirulent (incompatible or resistant host) interactions with plants (Clare et al., 2022). These effectors are recognized by the host with the help of R proteins encoded by resistance (*R*) genes with nucleotide binding site-leucine-rich repeats (NBS-NLR). Thus, a series of immune responses are triggered named Effector-Triggered Immunity (ETI) (*R* gene-based or vertical immunity), which is generally accompanied by the hypersensitive response (HR) and localized host cell death (Dolatabadian, 2020).

According to Chisholm et al., (2006), necrotrophic pathogens are evolved to suppress PTI response by releasing host selective toxins or necrotrophic effectors (NEs) that are small-secreted proteins. These necrotrophic effectors maintain pathogen recognition by host immunity receptors to manipulate the host to gain superiority (Vishwakarma et al., 2020; Wyatt et al., 2018). This is a typical inverse gene-for-gene interaction. Thus, the PCD immunity response is activated in the host, which helps the necrotrophic pathogen to feed on the dead cells. So, necrotrophic effector-triggered susceptibility (NETS) is induced by NE release (Friesen et al., 2007).

2.8 Barley-*Pyrenophora teres* pathosystem

The genetic makeup of resistance can be up to the isolate that is used since numerous races exist in *Ptt* and *Ptm*. Resistance against *P. teres* was indicated as both qualitative and quantitative according to Geschele, (1928). Up to date, over 340 QTLs conferring reactions to *Ptt* and over 140 QTLs for reactions to *Ptm* have been identified in previous studies (Clare et al., 2020). For *Ptm*, 36 loci were unique, and

5 of them were specific to *Ptm*, while the others were partially overlapping with *Ptt* loci (Clare et al., 2021).

Fungal effectors released by the pathogen can act as avirulence or virulence factors, as well as both at the same time. Effectors are needed for the pathogen to be recognized by the host and used by the pathogen for manipulating the host defense mechanism (Dahanayaka et al., 2022). Recent studies reported that the barley-hemibiotroph *P. teres* pathosystem likely follows both gene-for-gene and inverse gene-for-gene interactions (Pandey et al., 2021).

2.8.1 Genetics of barley reactions to *Pyrenophora teres* f. *teres*

Qualitative (gene-for-gene model) and quantitative resistance (complex genetic interactions) are found to be responsible for resistance against *Ptt* (Dinglasan et al., 2019). In this context, *Pt1*, *Pt2*, and *Pt3* were first identified as the effective loci for *Ptt* resistance (Mode & Schaller, 1958; Schaller, 1955). Later, the *Pt1* and *Pt2* loci collapsed with the *Rpt1* locus resulting in renaming it (Bockelman et al., 1977). Many more loci were renamed once more in the following years, although they had previous synonyms before (Clare et al., 2020). *Rpt1* (Bockelman et al., 1977) on 3H, *Rpt2* (Bockelman et al., 1977) on 1H, *Rpt3* (Bockelman et al., 1977) on 2H, *Rpt4* (Williams et al., 1999, 2003) on 7H, *Rpt5* (Manninen et al., 2006) (its current synonym is *Spt1* (Richards et al., 2016)) on 6H, *Rpt6* (Manninen et al., 2006) on 5H, and *Rpt7* (Franckowiak & Platz, 2013) on 4HL are the currently defined barley loci related to resistance against *Ptt*. *Rpt5* region, previously described as Pt_a locus (Manninen et al., 2006), is a complex locus, near the centromeric region of chromosome 6H but an important one for *Ptt*-barley interaction (Adhikari et al., 2019; Martin et al., 2018; Novakazi et al., 2019; Rozanova et al., 2019). Large region (*Rpt5* locus) implies to have tightly linked genes, that are dominant, recessive, and incomplete, playing roles in resistance to NTNB (Martin et al., 2018). Moreover, three genes/alleles (*Rpt5.f*, *Spt1.k*, *Spt1.r*) were described in this locus as responsible for resistance or susceptibility (Franckowiak & Platz, 2013; Richards et al., 2016).

The plant salicylic acid pathway is a defense pathway of barley at the early stage biotrophic infection cycle of *Ptt*. It is activated with pathogenesis-related proteins induced by the pathogen (Hassett et al., 2020). Tamang et al., (2021) identified a dominant resistance mechanism, likely as an example of gene-for-gene interaction, with a *R* gene and pathogen *Avr* gene playing roles at the early infection stage of *Ptt*. The researchers found that the *HvWRKY6* gene was taking a critical role in stopping the spreading of the pathogen at an early stage, right after the pathogen recognition. Although *Ptt* is a hemibiotrophic fungus, its biotrophic stage is short, and it is majorly a necrotroph in its infection cycle. During the infection stage, *Ptt* mostly feeds and infects as a necrotrophic fungus, growing intercellularly. At this stage, inverse gene-for-gene interaction occurs through NEs produced by *Ptt*. The necrotrophic stage induces ethylene and jasmonic acid pathways which take a role in upregulating defense-related genes (Hassett et al., 2020). Thus, host susceptibility is triggered through programmed cell death mediated by NEs (Pandey et al., 2021).

2.8.2 Genetics of barley reactions to *Pyrenophora teres* f. *maculata*

During its infection cycle, *Ptm* acts like a hemibiotroph, meaning that *Ptm* produces intracellular vesicles and feeds as a biotroph at first. Then, it turns fast into its necrotrophic form (Backes et al., 2021). Originally resistance-associated loci for *Ptm* were thought to be less complex than *Ptt*, since three major loci, *Rpt4* (Williams et al., 1999, 2003) on 7H, *Rpt6* (Manninen et al., 2006) on 5HS and *Rpt8* (Franckowiak & Platz, 2013) on 4HS, were found conferring seedling stage resistance. Other than these loci, *Rpt3* (Bockelman et al., 1977) on 2H, *Rpt5/Spt1* (Manninen et al., 2006) on 6H, *Rpt7* (Franckowiak & Platz, 2013) on 4HL are the currently defined loci related to the reaction against *Ptm*. Some of the loci associated with resistance were reported as taking a role in both *Ptt* and *Ptm* reactions in barley; however, it is unknown whether the same genes were responsible for the resistance or susceptibility reactions (Clare et al., 2020).

Since virulence effectors of *Ptm* are diverse, QTLs conferring resistance/susceptibility against the pathogen can be pathotype-specific (Alhashel et al., 2021). In a recent study done by Skiba et al., (2022), a dominant susceptibility gene at the *Rph4* locus on the 7H chromosome of barley was targeted by *Ptm*, virulent through Chr2 of the pathogen. Alhashel et al., (2023) conducted fine mapping to anchor this gene (later named *Sptm1*) into an approximately 400 kb region, offering a target for gene editing of the susceptibility gene.

2.8.3 Resistance against *Ptt* and *Ptm* in Turkish barley resources

There are some studies on the status of net blotch resistance in barley genotypes in Turkey. Most of these studies addressed the spot form of net blotch (Çelik Oğuz et al., 2016, 2017, 2019; Gerlegiz et al., 2014; Karakaya & Akyol, 2006; Taşkoparan & Karakaya, 2009; Yazıcı et al., 2015; Araya et al., 2022). These studies showed the variation in the net blotch resistance of barley genotypes in Turkey. Çelik Oğuz and Karakaya, (2017) found 24 pathotypes of *Ptt* and 26 pathotypes of *Ptm* in Turkey.

To date, there is only one study using association mapping for Turkish barley accessions, and zero using linkage mapping and QTL analysis. The mapping approach of association mapping population utilizes genome-wide association study (GWAS), allowing for finer-scale mapping of genes or QTLs. On the other hand, the mapping approach of a linkage mapping population uses linkage analysis, which is particularly useful in the initial mapping of genes and QTL mapping. In a recent study, Clare et al., (2021) used an association mapping population of Turkish wild and landrace barley genotypes to describe resistance loci against both forms of *P. teres*. The researchers used a population consisting of 102 wild barley genotypes (*H. spontaneum*) and 193 barley landraces (*H. vulgare*) gathered from various cultivation areas across Turkey to identify forms of resistance that might have been overlooked or lost through domestication. Thus, the researchers identified four loci against *Ptm* and ten against *Ptt*. Four of these loci (*QRpt-1H.1, QRpt-3H.1, QRpt-3H.3, QRpt-6H.1*) were potentially novel for *Ptt*. Moreover, two novel loci were

reported in *Ptm* interaction (*QRpt-5H.1* and *QRpt-5H.2*) which were previously described, *NBP_QRptt5-1* and *Qrptts-5HL.1*, respectively, in the*Ptt* interaction of barley. This study was important since landraces and wild barleys have the potential to be used as sources of genetic diversity.

2.9 Genotyping technologies

The beginning of plant genotyping was with the advances in molecular biology and the discovery of molecular markers in the early 1980s (Tripodi, 2023). A molecular marker is a DNA sequence with a known chromosomal location that is polymorphic among the individuals of a germplasm collection or population. Molecular markers are associated with a part of the genome and used to identify individuals or locate tightly linked genes of interest or QTLs. Thus, they provide a selection of the genotypes showing the desired trait that is hard to measure or relying on the developmental stage (Saeidnia et al., 2021). Since molecular markers are stable and easily detectable in almost every tissue, they have numerous applications in plant breeding and crop improvement areas (Tripodi, 2023). Potential applications of molecular markers are the identification of varieties for the protection of intellectual property rights of the breeders, genetic and association mapping, as well as studies on population genetics, conservation, and evolutionary genetics.

Molecular markers can be categorized according to the need for prior sequence knowledge, transmission mode (paternal/maternal organelle inheritance and bi-parental/maternal nuclear inheritance), loci number for each marker, their dominancy or codominancy, and analysis method such as hybridization-based, PCR-based, sequencing- and array-based techniques (Nadeem et al., 2018; Patel et al., 2015). Restriction fragment length polymorphisms (RFLPs) are the first-generation, hybridization-based molecular markers (Helentjaris et al., 1985). PCR-based or second-generation markers such as amplified fragment length polymorphisms (AFLPs), random amplification of polymorphic DNAs (RAPDs), simple-sequence repeats (SSR), sequence characterized amplified regions (SCARs), cleaved

amplified polymorphic sequences (CAPS), and inter simple sequence repeats (ISSRs) have been used for genotyping purposes (Deschamps et al., 2012). Nowadays, sequencing- or array-based SNP markers are often the marker of choice as third-generation molecular markers (Varshney, 2010).

2.9.1 RFLPs

RFLP markers rely on the usage of restriction endonucleases that digest the genome into random DNA fragments. Most RFLP markers are codominant and identify polymorphisms in restriction fragment lengths (Agarwal et al., 2008). Following the separation of fragments via gel electrophoresis, hybridization occurs to the probes in the southern blot that maintain their detection (Tripodi, 2023). To use probes, prior sequence knowledge is required, which creates a limitation (Agarwal et al., 2008). Since the process is time-consuming, second-generation PCR-based markers replaced RFLPs (Patel et al., 2015).

2.9.2 AFLPs

AFLP markers are based on the usage of primer recognition sites called adaptors that are ligated to the DNA fragments generated by the restriction enzyme. Then these fragments are selectively PCR-amplified (Agarwal et al., 2008). They are multi-locus markers, with no prior sequence information needed, which makes them useful for genetic mapping construction (Gebhardt, 2007). PCR products of AFLP are scored using gel electrophoresis or automated systems (Vuylsteke et al., 2007). The dominancy of this marker makes this marker disadvantageous due to the lower polymorphism levels obtained compared to the codominant ones (Garcia et al., 2004).

2.9.3 RAPD

In dominant RAPD marker technology, short arbitrary primers (approximately 10 bp) are used that are commercially available for arbitrary amplification of DNA sequences without requiring to possess previously available genomic information. A single primer for each reaction is employed, serving as both forward and reverse primers, binding at two different sites on the opposite DNA strands. Thus, changes such as gain or loss of the primer annealing regions create DNA polymorphism (Agarwal et al., 2008; Amiteye, 2021). Then PCR products, according to the presence or absence of the bands, are visualized using agarose gel electrophoresis. However, artifact occurrence is a problem of this method, since short primers may result in non-specific priming (Patel et al., 2015). Moreover, reproducibility of this marker is known to be low, due to its sensitivity to PCR conditions.

2.9.4 SSR

Highly polymorphic SSR markers (tandem repeated short DNA units) are 1-8 nucleotide length, which are abundant throughout the genome. They are reproducible, and transferable between species and codominant markers, making them advantageous (Patel et al., 2015). They also often possess multiple alleles per locus, but their uneven distribution through the genome limits their usability. Since SSR genotyping is gel-based, it is quite labor-intensive for numerous samples even for automated systems such as fragment analysis. SSR markers have less throughput and their cost per data is more expensive compared to the sequence-based systems (Kim et al., 2016).

2.9.5 ISSR

DNA sequences flanked by identical microsatellite repeats are amplified in the ISSR technique (Nadeem et al., 2018). This marker does not require a priori genomic

information (Tripodi, 2023). Dominant ISSR markers allow analysis of multiple loci and are mostly found in non-coding regions. They have advantages such as medium-reproducibility and high polymorphism, allowing to assessment of genetic diversity according to the presence/absence of the markers (Samarina et al., 2021). A single primer is used for each PCR reaction in this technique, unlike SSR markers in which two primers were occupied. Following PCR amplification, PCR products are loaded on agarose or polyacrylamide gel and visualized to score ISSR bands. A major drawback of the technique is the necessity of a sufficient quantity of high-quality DNA for each PCR reaction, which affects the uniformity and reproducibility of the bands (Amiteye, 2021).

2.9.6 SCAR

Mono-locus SCAR markers are derived from RAPD, AFLP, or ISSR markers (Amiteye, 2021). Polymorphic fragments of these markers associated with a target trait are cloned, sequenced, and primers are designed for the specific amplification of the region of interest. Although they are dominant markers, they can be converted to codominant markers with the help of tetranucleotide-recognizing restriction enzymes (Savitri, 2023). Their specificity and reproducibility are higher as compared to RAPD, AFLP, or ISSR markers, but less informative than the sequence-based ones (Bhatia & Bajwa, 2022).

2.9.7 CAPS

The original name for these markers was PCR–RFLP markers, which are codominant. PCR amplification primers are designed using available sequence information. Following the amplification of DNA, restriction enzymes are used to obtain fragments (Nadeem et al., 2018). At least two genotypes containing target DNA are aligned and checked whether SNPs create a restriction site corresponding to the restriction enzyme (Varshney, 2010). The main drawback of CAPS markers is

that they can only identify SNPs affecting the DNA-cutting capability of a restriction enzyme (Şahin et al., 2022).

2.9.8 SNP

Single nucleotide polymorphisms (SNPs), common DNA variants present across the genome of an organism, can be identified by genotyping (Baird et al., 2008). According to Rafalski, (2002), there is a SNP for each 48 bp in the non-coding sites of the maize genome, while a SNP for each 131 bp in the coding sites. In a study done by Zeng et al., (2009), SNP frequency was a SNP per 9 bp in the noncoding region of a barley gene, while a SNP per 10.7 bp in the coding one. SNPs are generally biallelic and found commonly in non-genic regions without impacting the phenotype of an organism (Ruff et al., 2020). SNPs can be evolutionarily neutral, but they can also be subject to natural selection if they affect the fitness of an organism. The evolutionary fate of a SNP depends on its location, its effect on the organism, and the specific environmental and genetic context. Many SNPs are neutral, meaning they are not subject to positive or negative selection. These often occur in non-coding regions of the DNA or result in synonymous mutations in coding regions (Edwards et al., 2007). On the other hand, functional SNPs occur in coding regions and change the amino acid sequence (nonsynonymous mutations), while regulatory SNPs in regulatory regions of genes (such as promoters or enhancers) can influence gene expression. Depending on whether the resulting change in gene expression is advantageous or disadvantageous, these SNPs may be subjected to selection. SNPs also can be in linkage disequilibrium. Namely, a SNP may be linked to another genetic variation that is under selection. In such cases, the SNP may appear to be under selection due to its association with the selected trait, even if the SNP itself is neutral. Genetic drift (random changes in allele frequencies) is another concept for SNP markers. Apart from selection, genetic drift can also affect the prevalence of SNPs in a population, especially in small ones. There are also population-specific SNP variants. In other words, some SNPs may be neutral in one

environment or population but could have selective value in another, depending on the varying environmental pressures or genetic backgrounds.

SNPs are known to be associated with differences in genetic traits such as disease resistance. To have a better comprehensive understanding of disease resistance mechanisms, SNP genotyping is a gold standard. SNPs can be used for investigating genetic similarity, identifying hybrid genotypes, constructing a genetic map, and association mapping. They are generally identified by disrupting restriction enzyme recognition regions and can be adapted to automated systems (Baird et al., 2008).

Transitions (substitution between purines (A, G) or pyrimidines (C, T)) and transversions (substitution between a purine and pyrimidine) are the two SNP forms (Bhatia & Bajwa, 2022; Patel et al., 2015). With the advent of array-based and sequencing technologies, SNPs paved the way for high-throughput genotyping (Savitri, 2023).

2.10 The sequencing technology

Sequencing is a method to identify the nitrogenous base order of a gene or genome and has important areas of usage in agriculture. DNA sequencing has been directed with the Sanger dideoxynucleoside chain termination technique (first-generation sequencing) which is based on preventing the extension of the growing DNA chain by the addition of dideoxynucleoside (Bao et al., 2014). In 2001, sequencing technology that was used in the human genome project was based on capillary electrophoresis of Sanger sequencing products. Back then, only 115 kbp (thousand base pairs) per day could be obtained by using one instrument, capable of running 96 reactions at a time (500-600 bases per reaction) (Mardis, 2011). In 2005, scientists succeeded in making significant progress in genomics with the advent of next-generation sequencing technologies (NGS, the second-generation sequencing; more formally called Massively Parallel Shotgun Sequencing – MPSS) that have replaced the chain terminator methods in most applications. The speed of this progress has

accelerated in the decade after the “Human Genome Project” was completed (Treangen & Salzberg, 2012). Two main challenges of sequencing are distinguishing misread nucleotides from real genetic variations and discriminating multi-copy genes from single-copy ones.

2.10.1 The basis of the chain termination technique

The initial step of the Sanger technique is the amplification of the DNA sequencing library by cloning nucleic acids into bacteria (*in vivo*) (Anderson & Schrijver, 2010; Bräutigam & Gowik, 2010). Then, the amplified DNA template is purified, and complementary strands of the DNA template are synthesized with DNA polymerase, primer, deoxynucleotides, and a low concentration of dideoxynucleoside triphosphates that does not contain 3'-hydroxyl group (Anderson & Schrijver, 2010; Javitt & Carner, 2014). The presence of this group is essential for incorporating incoming nucleotides via phosphodiester bonds to a nascent DNA strand on deoxyribose sugar. So, the absence of this group at the time of incorporation of fluorescently tagged dideoxynucleotides (ddNTP) results in termination. In other words, it means the cleavage of DNA polymerase from the chain as it gives an end of extension of nascent DNA. This termination process from different sites of nucleotides produces different lengths of DNA fragments. According to length difference, capillary electrophoresis is used to distinguish these varying lengths of DNA fragments (Børsting & Morling, 2015). At the end of the process, the ends of the fragments with a particular base give the information of the original DNA sequence.

Although the advances in Sanger sequencing such as labeling of each terminator nucleotide with a different color of fluorescent dye and usage of capillary gel electrophoresis helped to achieve an increase in throughput, there are still some critical problems with chain termination techniques (Deschamps & Campbell, 2010). First, it only obtains an average reading length of 1 kb pairs as a maximum (Anderson & Schrijver, 2010; Schadt et al., 2010). In addition, a relatively low number of

samples can be conducted per unit of time, and it consumes too much time and money. Moreover, *de novo* genome assembly cannot be simply performed in this technique (Anderson & Schrijver, 2010). The effects of these limitations could have been reduced with the invention of massively parallel sequencing engines called NGS technologies.

2.10.2 The basis of next-generation sequencing (NGS)

The first step of NGS is shearing the genome of interest into fragments with a size of ~500 bp. Afterward, two known sequences of oligonucleotides are ligated as adapters into both ends of the fragments, which let polymerase extend along the unknown fragment (Bahassi & Stambrook, 2014). Then, DNA template polonies are generated by PCR amplification as an *in vitro* cloning method for raising the signals (Bahassi & Stambrook, 2014; Bräutigam & Gowik, 2010; Schadt et al., 2010). These amplification and ligation steps help to select molecules that can be sequenced (Buermans & Den Dunnen, 2014). Following these steps, DNA template clusters are attached into a solid, sequenced with a phased approach, and imaged at the same time. After the base order assignment, reads are overlapped to align with the help of bioinformatics (Schadt et al., 2010). NGS platforms are composed of different technologies with the inclusion of Illumina, SOLID System, 454 Life Sciences, and third-generation sequencing techniques (Bahassi & Stambrook, 2014). However, 454 Life Sciences sequencing is completely over due to the high cost of reagents and the declaration of the firm, Roche, in 2016. According to this, Roche would no more supply or service 454 machines and chemicals. On the other hand, Illumina has become the major player among the NGS technologies and replaced 454 and SOLID due to its high efficiency and reasonable cost (Kulski, 2016).

The basis of Illumina technology is based on enzymatic reactions and imaging processes occurring inside a flow cell. The colony generation is performed in Illumina sequencing by solid-phase bridge amplification (Bahassi & Stambrook, 2014; Buermans & Den Dunnen, 2014). In the preparatory steps of Illumina, DNA

fragments are obtained and primers that are complementary to the adapters are immobilized to the inner plane of the flow cell channel. The double-stranded DNA fragments are denatured into single-stranded DNA molecules to attach the DNA libraries onto the flow cell and are hybridized with primers that are on the surface (Figure 2.1). Following the hybridization process, initial library molecules are removed, and polonies are generated from copied surface-suffixed fragments by isothermal amplification (Anderson & Schrijver, 2010).

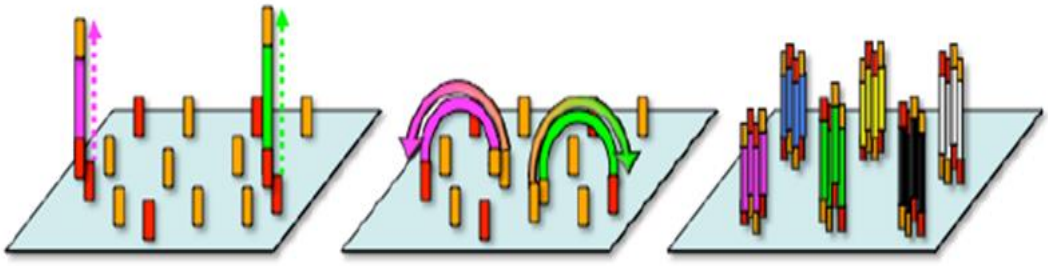

Figure 2.1. Illustration of isothermal bridge amplification in Illumina sequencing technology (Anderson & Schrijver, 2010).

During the amplification stage, a bridge structure is formed between the 3' end of the copied library molecules and complementary primers on the flow cell. Thereupon, one strand of DNA is removed either from forward or reverse surface primers that have a separable site, and all 3' ends are blocked with ddNTP terminators (Buermans & Den Dunnen, 2014). In this regard, Illumina is similar to the Sanger technique, with the usage of terminators; however, unlike the Sanger technique, these terminators let polymerization go on after fluorophore detection. Eventually, the sequence is determined with four-channel fluorescence scanning (Varshney, 2010).

Next-generation techniques have major advantages such as dramatically increased data throughput per run, and longer reads with an efficiency in cost and time (Berkman et al., 2012). This provided dense marker coverage and highly reliable mapping with high resolution (Huang et al., 2009). The advances in NGS technology methods opened the way for high-throughput sequencing of a wide spectrum of plant kinds (Anderson & Schrijver, 2010).

2.11 The genotyping techniques for SNP discovery

2.11.1 Whole-genome resequencing (WGS)

Sequencing for plant breeding can be performed at the level of regional or whole genome. Population size and genome coverage are important for obtaining informative markers, as well as the cost of the analysis (Xu et al., 2012). Although WGS is informative for genomes of some crops, genomes over 1 Gbp and highly repetitive ones are not well suited to this method such as wheat (Hamilton & Robin Buell, 2012). Since whole genome sequencing is priceful, instead of individual genome sequencing, 'pool-seq' can be used providing pools of individuals to be sequenced at reasonable costs (Futschik & Schlötterer, 2010). Rellstab et al., (2013) reported that pooled whole-genome resequencing maintained read coverage over 20x. On the other hand, techniques using restriction enzymes for constructing reduced genome complexity (e.g. GBS) or sequence capture (e.g. SNP-array) can be used to obtain partial genome representation libraries cost-effectively.

2.11.2 Sequence capture

Sequence capture is a genomic enrichment (genome-subsampling) strategy for sequencing broad or specific targets such as exome or genomic regions related to a trait efficiently and cost-effectively (Ray & Satya, 2014). Target enrichment methods such as hybridization-based, PCR amplification-based, or molecular inversion probe (MIP)-based amplification, are used as a sequence capture technique (Mamanova et al., 2010).

In hybridization-based methods, libraries are constructed from random fragments of DNA by modifying with oligonucleotides (adapter ligation) containing the binding region for the polymerase for interfacing with the sequence equipment. Before sequencing, they are hybridized to custom baits which are biotinylated oligonucleotide probes (Harvey et al., 2016). Hybrid capturing can be based on

solution phase suspended beads with baits or solid phase bound baits (SNP arrays) which are not easy to customize (Andermann et al., 2020).

However, solution-based hybridization capture systems are customizable. Biotinylated and free DNA or RNA baits provide selecting targeted fragments-bait hybrids using streptavidin-coated magnetic beads in solution. The captured DNA library is then amplified via PCR for sequencing (Gasc et al., 2016). In PCR amplification-based enrichment, the RainStorm platform, developed by RainDance Technologies, is used which is based on microdroplets as in emulsion PCR. Each microdroplet is responsible for a single PCR by containing a single primer pair, genomic DNA, and other PCR reagents. Thus, around 4,000 primer pairs can be used for simultaneous PCR amplification for target enrichment (Mamanova et al., 2010). Whereas in MIP-based amplification, oligonucleotides consisting of a common linker sided by target-specific sequences anneal to the target and circularized by using ligase. Following the digestion of genomic DNA, circularized one is PCR-amplified with the help of primers at the linker. Then sequencing is performed (Mamanova et al., 2010).

SNP arrays containing haplotype-specific SNPs or tag-SNPs can be used to provide enough information based on SNPs in proximity or linkage disequilibrium (LD) (Andermann et al., 2020). There are SNP arrays specifically designed for numerous crops such as wheat (Winfield et al., 2016), rice (Chen et al., 2014), maize (Ganal et al., 2011), and barley (Comadran et al., 2012). Close et al., (2009) designed an array-based platform for barley constituting SNP markers with the development of Illumina GoldenGate assays. Then, Comadran et al., (2012) developed the barley 9K iSelect SNP Chip with existing 2,832 barley markers obtained from GoldenGate assays, and 5,010 additional markers newly discovered through Illumina RNA-seq. In 2017, Bayer et al., (2017) reported developing a barley array called 50k Illumina Infinium iSelect constituted of markers from previous platforms, as well as 42,316 new markers discovered through exome capture analysis of selected barley lines using the barley reference genome (Beier et al., 2017). The last array technology developed for barley is Illumina Infinium Wheat Barley 40K SNP array Version 1.0

constituting 25,363 wheat-specific and 14,261 barley-specific SNPs (Keeble-Gagnère et al., 2021).

Arrays have pros such as providing less genotypical error and having less missing data as compared to the reduced representation sequencing techniques. The cost-effectively discovered markers are widely studied for genome-trait association studies, molecular characterization of germplasms, and MAS (Keeble-Gagnère et al., 2021). Besides their pros, there are cons of arrays such as creating SNP ascertainment bias due to SNP selection developed from a small number of genotypes while designing the arrays (Geibel et al., 2021). Furthermore, although a dot plot is obtained in SNP arrays at the end of this hybridization-based technique, true allelic states may not be reflected in case heterozygous loci exist. Moreover, the resolution of the maps may not be sufficient when using a low number of markers and there is a limitation of array-based markers' mostly being chosen within genes (exonic regions) (Abed et al., 2022). Because array-based technologies are often designed to target specific regions of the genome that are more likely to be functionally relevant (informative), this may create a limitation for taking advantage of the markers within the non-coding regions. In addition, customizing of the SNP arrays is not possible, which does not offer flexibility to add markers and limits the number of markers compared to the sequencing technologies (Eagle et al., 2021).

2.11.3 RNA sequencing and exome sequencing

Protein-coding regions, which are 1%–2% of the whole genome of an organism, are focused on in both RNA sequencing and exome sequencing techniques. These regions have numerous functional variants, while are scarce repetitive regions (Scheben et al., 2017). Biologically significant SNPs can be detected using sequencing on transcriptome base (RNA-seq). Complementary DNA is sequenced in RNA-seq for obtaining sequences of transcriptome and quantifying levels of RNA transcripts. RNA-seq is used for SNP genotyping purposes besides gene expression studies without the need for prior genomic information. However, expression tissue

and time affect the transcript abundance in this method, generating a limitation in its usage (Cirulli et al., 2010). Besides, the need for high-quality samples and rapid processes due to quick mRNA degradation may be challenging. Moreover, a limited number of SNPs in coding regions may not be enough analyses such as GWAS (Scheben et al., 2017).

The total of all exon sequences throughout the genome is called as exome. Following DNA fragmentation, and probe hybridization, magnetic streptavidin beads or arrays are used to bind the probes in exome sequencing. Then, unbound DNA fragments are washed away, and bound ones are amplified through PCR and sequenced. Thus, unexpressed alleles and genes that are hindered in RNA-seq can be analyzed while scaling targeted capture up to thousands of genes. However, this technique is highly dependent on the existence of a well-annotated reference genome not to lead to missing data. Furthermore, it is quite an expensive analysis compared to RNA-seq (Scheben et al., 2017).

2.11.4 Reduced representation libraries (RRL)

Van Tassell et al., (2008) developed a technique called RRL to digest pooled DNA samples using a frequently cutting restriction endonuclease, followed by ligation of barcoded adaptors and their amplification via PCR. Then the fragments are size-selected and sequenced for polymorphism detecting the pool of individuals, not the individual itself. The major drawback of this technique is the necessity for a high amount (approximately 5 μg) of DNA for each pool.

2.11.5 CRoPs™

In complexity reduction of polymorphic sequences (CRoPs™) technique, tagged complexity-reduced libraries are constructed using AFLP. Followed by PCR, the products are sequenced using a Genome Sequencer (GS) 20/GS FLX NGS system. Following clustering the sequences and aligning them, SNP mining is performed via

bioinformatics analyses. Thus, many organisms can be genotyped at medium or large scale. There are two restriction enzymes in CRoPS^{TM} (Van Orsouw et al., 2007). Following complexity reduction, short barcode (different for each DNA sample) identifier sequences or multiplex identifier sequences (MIDs) on the sequencing platform are incorporated into the ligated adaptors, and AFLP reads are processed and mined for SNP variants. This technology is patented by "Keygene". Using CRoPs^{TM}, the DNA amount per sample could be lessened to around 100–500 ng.

2.11.6 RAD-seq-based genotyping: sdRAD, ddRAD, BestRAD and 2bRAD

Restriction-site Associated DNA (RAD) tags are sequenced in this technique to discover SNPs adjacent to restriction enzyme sites. There is single-digest in RAD-seq (sdRAD) (Baird et al., 2008). Following restriction enzyme cuts genomic DNA, and an adapter containing amplification and sequencing primers which is complementary to the restriction site is attached for barcoding. Mechanical fragmentation selects fragment sizes using a DNA sonicator in the original technique (Andrews et al., 2016). The size selection stage provides obtaining fragments in the wanted size for assurance of sequencing efficiency (Chambers et al., 2023). During the size selection stage, a second restriction enzyme can be used instead of the fragmentation, which provides an improved size selection of the fragments. This version of the method is called as double-digest RAD-seq (ddRAD) (Peterson et al., 2012). Then, the selected fragments are PCR-amplified and sequenced as double-end reading (Kess et al., 2015). Marker density or genome coverage can be arranged according to the type of used restriction enzyme and may provide the reduction of missing data (Ulaszewski et al., 2021). Still, DNA quality limitations exist in ddRAD which is a more critical issue than in the capture methods (Suchan et al., 2016).

RAD combined with bait-based (biotinylated DNA or RNA molecules) capture step for targeted sequence enrichment (sequence capture) is termed as 'BestRAD', 'Rapture' or 'RAD capture'. BestRAD uses biotinylated adapters for extracting restriction site-associated DNA. BestRAD uses a single digest and sonication, as

well. Thus, the technique decreases the expenses for sequencing and library construction as compared to RAD (Ali et al., 2016). Heterozygotes can be genotyped more accurately with a more repeatable set of marker data using this technique compared to RAD (Bekele et al., 2020).

On the other hand, Type IIb restriction enzymes are used in 2bRAD for cleaving DNA on either side of the recognition region to generate constant short lengths (33–36bp) of fragments. Due to short fragments, there is no need to perform paired-end sequencing (Andrews et al., 2016). In addition, since uniform fragments are generated, the size-selection step is not needed in 2bRAD. Restriction site-selective adaptors are used for the ligation of these fragments with their overhangs. Then, the fragments are amplified and sequenced (Wang et al., 2012). Unfortunately, the multiplexity of this technique is not as high as other reduced representation techniques such as GBS. In RAD, 96 samples can be worked on, while up to 384 in GBS. Although 2bRAD increases this number with the help of indexed PCR primers usage, preparing each sample into a single library is neither cost-effective nor labor-effective (Guo et al., 2014).

2.11.7 Genotyping-by-sequencing (GBS)

The GBS method was initially reported by Elshire et al. (2011) and built upon the protocol of RAD tags (Peterson et al., 2014). There are two background events of GBS. The first one is target enrichment and the second one is restriction enzymes. In the former, specific genes or genomic subsets are amplified via PCR and inversion probes are used for hybridization (array). In the latter, methylation-sensitive restriction enzymes (RE) cleave DNA at unmethylated cytosine residues and are used for targeting genes and filtering out repetitive genomic fractions (Favre et al., 2021). Although there is one restriction enzyme in the original GBS protocol, this technique was modified to the version of two enzymes to reduce the complexity reduction. Thus, this method with double enzymes has a more uniform library construction compared to the previous version (Bhat et al., 2016). REs frequently

used for crops are *ApeK*I, *Pst*I, and *EcoR*I combinationally used with enzymes like *Msp*I, *Mse*I, and *Hpa*II (Favre et al., 2021).

First, the GBS library needs to be constructed in the GBS pipeline. Adapters with barcode sequences are ligated to the sticky ends caused by restrictions for sequencing the library (Susmitha et al., 2023). Barcode adapters need to be designed with several criteria. It is important that barcode sequences do not have very high or low GC content or not recreate an enzyme recognition site (Johnson et al., 2023). Moreover, balance across some different nucleotides should be maintained as much as possible at all positions of the barcode for optimal nucleotide detection using red (for A/C) and green laser (for G/T). Moreover, barcode pairs should be designed with at least three differing nucleotides to compensate and correct for a single base call error (Somervuo et al., 2018). The major drawback of Illumina sequencing is incorrect phasing issues that may result in the reduction of read quality and calling incorrect bases due to the fragments falling out of phase (either a base ahead or behind). To solve this problem in GBS, variable RE cut sites should be designed with variable length barcode sets so as not to lose signal intensity during reading (Elshire et al., 2011).

Second, selected library fragments need to be amplified via PCR using primers common to the restriction sites (Figure 2.2). The template to be analyzed is prepared fully automatically, and DNA samples are sequenced in an NGS platform resulting in single-end reads while standard Illumina generates data from both ends (paired end read) of the library fragment. Before sequencing, the formation of adapter dimers, seen only in 0.05% of the raw sequence, can be controlled by adapter titration. Filtration is performed as soon as the raw sequence data is obtained. Then, it is aligned to a reference genome usually using the Burrows-Wheeler alignment tool (BWA) or Bowtie 2 generating SAM (Sequence Alignment Map) or BAM (binary version of SAM) files (Kim et al., 2016). Finally, SNPs are identified from aligned tags (alleles), and they are scored for their depth and coverage which can be controlled with enzyme choice. Sequence coverage, which is dependent on enzyme choice and multiplex level, was found highly reproducible in the range of 20x and

40x for identifying loci (Favre et al., 2021). Moreover, aligning to a reference genome (a complete genome sequence) increases the number of SNPs discovered. TASSEL-GBS, Stacks, Fast-GBS, and IGST are used for analyzing the GBS pipeline using a reference-based approach, while UNEAK and Ustacks are used for *de novo* ones (Torkamaneh et al., 2017).

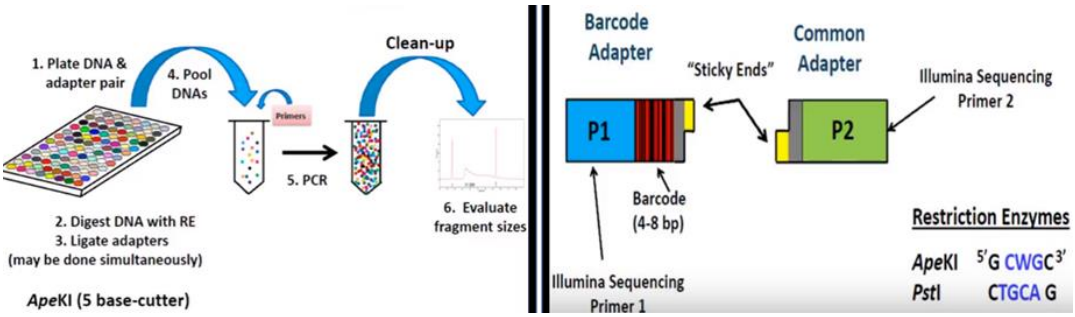

Figure 2.2. Library construction stages of GBS, its adapters and enzymes (Elshire et al., 2011).

The potential applications of the GBS technique are marker discovery, MAS, population genetics, germplasm characterization, linkage mapping, association mapping, fine-mapping, bulked segregant analysis, genomic selection, and improving reference genome assembly (Karaköy et al., 2023). GBS provides reduced sample handling, an efficient barcoding system, and high numbers of SNP calling (He et al., 2014). Additionally, non-exonic markers can be mapped via GBS unlike exome capture, which gives an advantage in QTL identification since the majority of trait-associated markers are found in non-coding genomic regions (Tanaka et al., 2019). The other major advantage of GBS is its multiplexity with the help of adapter sequences so that multiple DNA samples (from 96 to 384) can be genotyped at the same time cost-effectively (Huang et al., 2014; Poland et al., 2012). In comparison, library construction involves fewer steps in GBS than the other SNP genotyping platform, called RAD in which a restriction enzyme is used for reduced genome representation. Moreover, less DNA is required in GBS as compared to RAD with no need for a size selection stage (Wickland et al., 2017). On the other hand, SNP arrays need prior knowledge of the SNPs involved to design the probes, while GBS does not (Clarke et al., 2016). Moreover, there is a minimization of ascertainment

bias in GBS, due to the absence of a fixed design, unlike arrays (Lachance & Tishkoff, 2013).

Challenges of GBS are difficulties in bioinformatic analysis and missing data that needs imputation (Ruff et al., 2020). The most significant issues regarding GBS are DNA quality and correct quantification. So, it is vital to obtain DNA samples with this in mind (Öncü Öner et al., 2023). Obtaining high-quality DNA, accurate quantification using an intercalating dye instead of spectroscopy, and accurate liquid handling are essential in GBS analysis. Despite the challenges, it is proposed that GBS will be a key component for genotyping in breeding programs in the future.

2.11.8 DArT-seq as a GBS technique

Diversity Array Technology (DArT) markers are based on differential hybridization using a microarray for detecting variations derived from substitutions (SNPs and InDels) at restriction sites (Deres & Feyissa, 2023). DArT works well even for polyploids (Kilian et al., 2012). It uses restriction enzymes (e.g. *PstI* and *MseI*) for a reduced representation of the genome (Hassett et al., 2023). Then, adapters are ligated, followed by PCR amplification of the adapter-ligated fragments and subsequent hybridization of fluorescently labeled DNA fragments to an array with a library of the investigated species. Thus, informative DArT markers are obtained that are polymorphic (Appleby et al., 2009) (Figure 2.3).

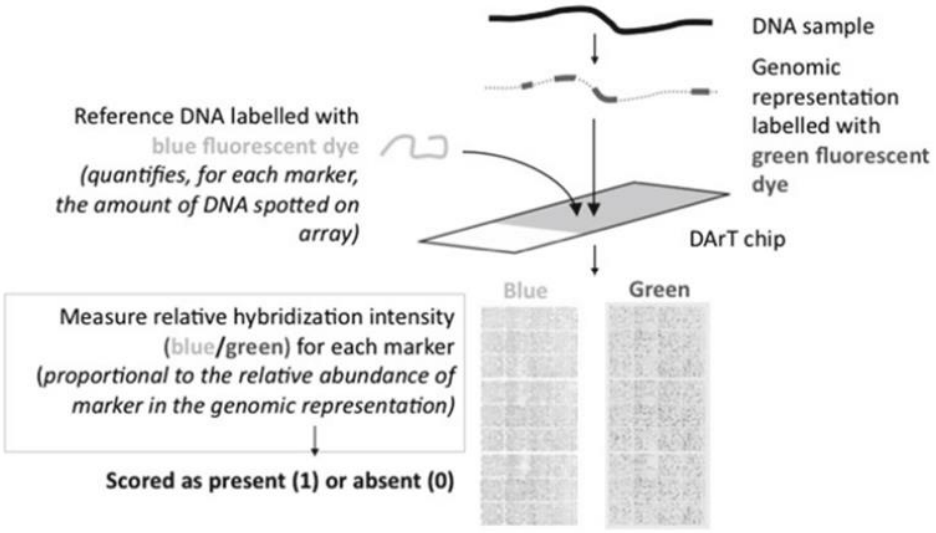

Figure 2.3. Illustration of DArT assay (Kilian et al., 2012).

The DArT technique has been adapted into a GBS-based version, called DArT-seq. SNPs and silicoDArT markers (presence or absence of restriction fragments in the representation) are obtained in this technique (Hassani et al., 2020). SNP markers are codominant markers that can distinguish heterozygous from homozygous, while silicoDArT markers are dominant markers based on methylation variation (Mudaki et al., 2023). DArT-seq targets low-copy-number sequences separating them from the repetitive regions with the help of used restriction enzymes. Thus, genomic complexity is reduced by selecting the genomic fractions primarily related to the coding regions of active genes and subsequently sequencing them (Allan et al., 2020).

DArT-seq is a multiplex technique, meaning that 96 samples can be sequenced in a single lane of the sequencing machine (Crossa et al., 2016). It ensures higher sequencing depth and uses strict filter criteria. This results in higher marker density in a cheaper way with broader genomic coverage and less missing data compared to DArT, as well as other GBS techniques (Allan et al., 2020). Thousands of genomic loci are tested in a single DArT assay, producing up to thousands of markers (Kilian et al., 2012). In a typical DArT assay, almost 200,000 fragments are sequenced ten times with an average number of ~2,000,000 sequence tags (barcodes) for each

sample (Valdisser et al., 2017). It performs single-end reading and gives sequences of 69-77 bp in length using a HiSeq2000 or HiSeq2500 platform (Illumina, USA) (Hassett et al., 2023). Marker metadata derived from representative DNA samples of the analyzed population is produced and markers are further selected using the DArTsoft pipeline (DArT P/L, Australia) (www.diversityarrays.com). The suspicious SNP variants are eliminated according to their call rate, sequencing depth, and reproducibility. Thanks to the construction of multiple libraries of the same individual, reproducibility is scored as most samples are processed as duplicates (Li et al., 2015). So, DArT-seq provides large numbers of handful markers ready to be used (Kilian et al., 2012).

Read depth (sequencing depth or depth of coverage) is a crucial concept in NGS applications. It defines how many reads are obtained per a specific nucleotide in the sequenced region, during the process of sequencing. Its highness gives confidence in calling a variant at a specific location. In a recent study based on DArT-seq, the average read depth was found as 18 for SNPs (threshold was 7), while 17.2 for silicoDArTs (threshold was 8) (Li et al., 2015). DArT-seq, as a SNP genotyping technology, has much to offer for plant breeding studies in terms of providing high read depth, multiplexity, and cost-effectiveness.

2.12 Some applications of genotyping methods

2.12.1 Linkage (genetic) mapping and QTL mapping (QTL-IM)

Segregation of alleles of one gene is independent of the other ones according to Mendel's second law of independent assortment (random separation of chromosomes into gametes during meiosis). Although this is valid for genes located on different chromosomes; it may not be the case for the genes on the same chromosome. Therefore, when two genes are close enough together, they may be linked and not independently segregated in meiosis. So, genes in the linkage groups act like or are inherited as a unit, rather than independent at this stage. At the prophase of meiosis-

I, recombination (crossing over) occurs which is the genetic material exchange between the parents, forming non-parental gene combinations. The possibility of crossing over, and producing recombinant genes, is associated with the distance between two genes. The further they are situated on a chromosome, the higher the frequency of recombination.

Linkage mapping (genetic mapping) is used for determining the relative distances between markers by calculating their recombination frequency. It provides dissemination of the molecular markers into their linkage groups. The number of the linkage groups throughout the genome needs to be corresponding to the total chromosome number. Thus, genetic positions and orders of markers on the chromosomes are portrayed in linkage groups throughout the whole genome (Abed et al., 2022). CentiMorgan (cM) is the unit of the linkage map, in which recombination frequency between two loci is 1% chance in a linkage group (Şahin et al., 2022). Linkage mapping is performed before conducting QTL mapping to ease the QTL analysis.

QTLs are the genomic regions effective on quantitative traits, which can be tagged by molecular markers. High marker density ensures obtaining a high-resolution mapping. Methods such as GBS and DArT-seq, producing high numbers of markers, are used for genotyping the mapping populations for QTL identification. *In silico* analyses, based on phenotyping and genotyping data derived from the mapping population, are performed to construct QTL maps. QTL analysis identifies major or minor QTLs playing roles in the trait of interest (Scheben et al., 2017).

During QTL analysis, standard interval mapping (SIM or IM) and multiple imputation (IMP) methods provide information when a single QTL is unlinked, while composite interval mapping (CIM) for both linked and unlinked QTLs. To calculate the linkage between markers, the ratio of linkage versus no linkage is calculated. This value is called as odds ratio or logarithm of odds (LOD) showing whether using either of these mapping methods' performance is acceptable. Generally, a LOD score of 3.0 or above is accepted as significant or ideal for

identifying QTLs (Riaz et al., 2021). The main genes underlying QTLs associated with the trait of interest can be cloned for various breeding purposes such as gene pyramiding.

To obtain a high-resolution map, parental genotypes that are phenotypically and genotypically diverse (showing distinct allelic variations and a certain level of polymorphism in terms of a target trait) are selected for producing a segregating population. The mapping population is generally constituted of 50–250 individuals (Nadeem et al., 2018). Different types of mapping populations that are family-based (bi-parental or multi-parental) can be used for QTL mapping. Hybrid populations (such as second generation (F_2) populations and first backcross (BC_1)) or homozygous immortal lines (such as backcross inbred lines (BILs), near-isogenic lines (NILs), recombinant inbred lines (RILs) and doubled haploid (DH) populations) can be used for QTL analysis (Alqudah et al., 2020).

NILs are the lines that have the same genetic structure except for a single or several loci letting identification of the markers linked to the interested trait (Mia et al., 2019). They are obtained through several (7-8) rounds of backcrossing and subsequently selecting the individuals showing the desired trait at each backcrossing and selfing them for maintaining the homozygosity at the specific locus/loci (Rafalski et al., 1996). Likewise, RILs are obtained by multiple (7-10) generations of inbreeding (selfing) or sibling mating from the progenies of F_1 until fixing the whole alleles (Wu et al., 2007). On the other hand, DH plants can be obtained from early hybrids (F_1 or F_2) in a single generation, which is advantageous compared to the other homozygous immortal lines in terms of time required for the construction of the population.

2.12.2 Linkage disequilibrium-based association mapping (AM)

Linkage disequilibrium (LD) is the statistical association of alleles at different loci in members of a population (Slatkin, 2008). Association mapping (AM) based on

linkage disequilibrium evaluates the frequency of alleles of genetic variants across individuals within the mapping population on a genome-wide scale (Scheben et al., 2017). AM benefits from using a genotyping set representing lots of ancestral recombination cycles and possessing high genetic variability due to the high number of alleles at each locus (Gupta et al., 2014). Thus, alleles can be identified using AM thanks to the large population genetic background, producing high-resolution maps (Nadeem et al., 2018).

Historic germplasms, breeding populations, and one or more family-based (biparental or multiparental) mapping populations can be used for AM. Various mating designs have been offered to construct multi-parental mapping populations such as quadri-parental populations using four parents for hybridizing in a half diallel. The other two designs are multi-parental advanced generation intercrosses (MAGIC) and nested association-mapping (NAM). MAGIC is the immortal fixed lines that are mosaics of 4, 8, or 16 parents. The MAGIC population can be obtained through biparental hybridization at the beginning and subsequent hybridization between F_1s or mating inbreds of the parents for subsequent generations (Arrones et al., 2020; Gupta et al., 2014). On the other hand, the NAM population is based on independent RILs obtained by crossing several parental inbreds with a common parental inbred (Gireesh et al., 2021).

In the pipeline of AM, individuals of the mapping population are first selected. Then they are phenotypically and genotypically analyzed. Through *in silico* analyses based on phenotypic and genotypic data of the population, the level and influence of the population structure are evaluated using a mixed-model approach, called a general linear model (GLM) or mixed linear model (MLM). The kinship matrix (K) and population structure (Q) are investigated in MLM, while only population structure is used as a covariate in GLM (Saeidnia et al., 2021). So that, molecular markers associated with the trait of interest can be identified. Moreover, linkage mapping can be combined with association mapping studies. Thus, the physical positions of markers flanking QTLs are translated into genetic positions to be further used in MAS (Abed et al., 2022).

Although AM has many advantages due to the high numbers of recombination events, the mapping results may be distorted depending on the population structure of the used lines (Gage et al., 2020).

2.13 Doubled haploidy technique as a method for obtaining DH plants

Conventional breeding procedure takes at least six generations to achieve almost complete homozygosity of the plants. The advent of doubled haploidy technologies in the 1980s provided complete (100%) homozygosity through a single generation, offering easier phenotyping of genotypes in multiple replicates (Tuvesson et al., 2021). DHs or pure lines can be obtained in a year using *in vivo, in vitro*, or a combination of both methods (Devaux et al., 1995).

Early-generation hybrids (F_1 and F_2), that go through only a single or two recombination events, can be used to obtain DHs. The recombination events are fixed in the population using doubled haploidy techniques, creating immortal lines. On the contrary, the RIL population has numerous rounds of recombination before the recombination events get fixed. So, DHs are useful for the construction of mapping populations in a fairly short time to perform QTL analysis (Weyen, 2021). To produce DHs, microspores, anthers, ovaries, ovules, and flower buds can be used in *in vitro* culture techniques. In *in vivo* techniques, haploid inducer lines are used, while intra-specific or inter-specific (wide crosses) crosses are based on the combination of *in vitro* and *in vivo* methods (Seguí-Simarro et al., 2021).

There are two methods in intra-specific crossing to obtain DHs; (i) pollen treatment using irradiation or chemicals before pollination, resulting in haploid maternal embryos generally needing embryo rescue *(in vitro)*, and (ii) haploid inducer line usage producing maternal or paternal haploid embryos in only few species. To do an intra-specific cross using the diploid haploid inducer line and the tetraploid potato cultivar (possessing a different chromosome number than the inducer), they are crossed to produce viable seeds containing haploid embryos. They then turn into

dihaploids. Although they are not homozygous, they can be used for easier mapping studies allowing working at the diploid level, or be used for inter-specific crossing to obtain DHs. The other method of obtaining *in vivo* DHs, used in maize, is based on mutating gametophyte development and double fertilization genes for making the maize lines haploid inducers (Seguí-Simarro et al., 2021).

In the inter-specific crosses technique, a cultivar, and its wild relative are crossed (only applicable to some crops) and chromosomes of one of the parents spontaneously get eliminated due to an unstable joint of genetic materials. A wild relative of the crossed cultivar (e.g. *Hordeum bulbosum* for barley) is used as the male parent. Then the haploid zygotic embryo is rescued for its *in vitro* regeneration and DH plant generation eventually (Cistué et al., 2011).

Female or male gametes can be used for obtaining DHs based on *in vitro* methods. Using male gametes to produce DHs is called androgenesis while using female ones is gynogenesis. In gynogenesis, an embryo sac (generally haploid) is obtained to develop a gynogenic embryo from egg cells, and chromosome doubling treatment is applied to induce the transition of cells from haploidy to doubled haploidy (Seguí-Simarro et al., 2021). In microspore or anther culture, microspores (isolated from anthers) or anthers of F_1 or F_2 used as the male parent can be employed as a source of gametophytic cells, evolving to haploid embryoids and finally to DH plantlets (Bélanger et al., 2016).

2.14 Construction of barley DH mapping population using anther culture

The anther culture stages of barley include the pretreatment of anthers containing pollen cells. Pretreatment of anthers means applying stress factors to them. It is required for switching microspores from gametophyte to sporophytic stage that helps increase regenerated plant number per anther. Cold exposure and/or mannitol (sugar starvation) are used for the pretreatment of barley anthers (Devaux & Kasha, 2009). Then, the anthers are placed onto an induction medium for the formation of

embryoids/calli. After incubation of the anthers, transferring anthers into regeneration, and subsequently to rooting media, provides generation of regenerant plants with roots. Normally, haploid plants are sterile and do not produce seeds. Therefore, chromosome-doubling chemicals are used for plants that do not go into a stage of spontaneous haploid doubling. However, barley anther culture lets obtain DHs with spontaneous chromosome doubling (almost 70%) without the need for chemical usage like colchicine (Khan et al., 2022).

Apart from culture conditions and nutrient medium ingredients, stress-free donor plant usage is also important for the efficiency of the barley anther culture. Therefore, culture rooms are used for growing the donor plants to maintain controlled environmental conditions such as temperature, humidity, and day length (Ohnoutkova et al., 2019). The development stage of the pollen cells also matters for starting the anther culture, which can be controlled by checking the anthers under a microscope (Ahmed et al., 2021). Mid- to the late uninucleate stage of microspores is known as suitable to start barley anther culture (Devaux & Kasha, 2009). Besides, the anther culture recalcitrant varieties affect the success of callus formation and obtaining green regenerants (Ahmed et al., 2021). Producing green regenerants is an important issue since albino plantlets lacking chloroplast creates a limitation for the technique. Albino plants cannot do photosynthesis which is crucial for their survival. Even though there are challenges in this technique, anther culture is a golden standard due to its simplicity and efficiency for *in vitro* generation of barley genetic mapping population (Mishra & Rao, 2016).

CHAPTER 3

MATERIALS AND METHODS

MATERIALS

Plant materials

A barley doubled haploid mapping population was generated using anther culture technique derived from F_2 donor hybrid plants of varieties, Avcı 2002 and Bülbül 89 which are known to be moderately resistant and/or moderately resistant-moderately susceptible and susceptible and/or moderately susceptible-susceptible to NTNB disease, respectively (Araya et al., 2022; Çelik Oğuz et al., 2016; Yazıcı et al., 2015). Avcı 2002 is a six-rowed Turkish barley cultivar, while Bülbül 89 is a two-rowed one. Both cultivars were developed by the Field Crops Central Research Institute (FCCRI) in Turkey (www.arastirma.tarimorman.gov.tr). Avcı 2002 was represented as "A" and Bülbül 89 as "B", while the DH population was designated as "A × B DH".

Fungal isolate

The virulent single spore isolate of *Pyrenophora teres* f. *teres* (*Ptt*) GPS18, collected from barley (*Hordeum vulgare* L.) fields in Sivas, Turkey (Çelik Oğuz & Karakaya, 2017), was used in the phenotypical characterization of the plants. The *Ptt* isolate was kindly provided by Prof. Dr. Aziz Karakaya and Assoc. Prof. Dr. Arzu Çelik Oğuz (Ankara University, Turkey).

METHODS

3.1 Generation of A × B DH mapping population

3.1.1 Cultivation of maternal and paternal genotypes

The elite seeds (pure, selected) of Avcı 2002 and Bülbül 89 barley varieties were used as the sources of maternal and paternal genotypes for hybridization. These seeds were planted into the pots as five seeds per pot in three different periods with a fifteen-day interval. The plants were grown in the plant growth rooms in FCCRI, Biotechnology Research Center with a temperature of 24±2°C, and 80% relative humidity under a 16 hour of light photoperiod until they produced at least 2-3 leaves (Perea-Brenes et al., 2022). Then they were transferred into the plant growth chambers (SANYO Versatile Environmental Test Chamber, Japan) maintaining vernalization conditions (at 6°C and 8 h of light photoperiod for 4-6 weeks) as per their vernalization requirements (Cha et al., 2022). They were transferred into the greenhouses in FCCRI and grown until their harvest time.

3.1.2 Generation of F_1 hybrid plants

Hybridization studies were carried out in the greenhouses located at the FCCRI. The "Egg-topping" emasculation method (Jensen, 1977) was used for hybridization as described by Thomas et al., (2019). The Avcı 2002 variety was used as the maternal source of hybridization. Since barley has male and female organs carried in its spikelets (barley flowering units) on a spike, the spikes of cv. Avcı 2002 were emasculated for preparing the maternal genotype. The right development stage for emasculating the spikes was after awns started to emerge from the flag leaf auricles, and when the anthers were still green. During the emasculation process, one-third of the spikelet top was cut and three anthers within the spikelets were removed using a thin-tipped forceps. Then, emasculated spikes were isolated with a paper bag to

prevent unwanted pollen intake from the outside. They were rested for three days to let the stigmas get ready as their spikelets were open for pollination.

The pollen donor spikes were treated similarly. Spikes with mature (yellow) anthers were harvested from cv. Bülbül 89, which was used as the paternal source of hybridization. The upper portion of the collected florets was cut with fine-tipped scissors for the easy shedding of the pollens. Spikes that were ready to release their pollens were rapidly closed and moved towards the opening of the paper bags of the maternal plants. Pollinated spikes were re-covered with the paper bag. Paper bags with hybrid seeds were collected during the harvesting time of the mature crop. The obtained Avcı 2002/Bülbül 89 (A × B) hybrid seeds were kept at a temperature of 4°C until sowing.

3.1.3 Generation of F_2 hybrid plants

The F_1 seeds were planted in vials containing soil-perlite mixtures with 2-3 seeds per viol (Figure 3.1). The F_1 plants were maintained in a plant growth room in FCCRI, Biotechnology Research Center with a temperature of 24±2°C and 80% relative humidity for 16 h of light photoperiod. Ten days after the germination of seeds, the plants were vernalized for 4-6 weeks at 6°C and 8 h of light photoperiod in the plant growth chambers (Figure 3.2). Then, they were taken to the controlled growth room with larger pots, and foliar fertilizer, insecticide and fungicide were applied approximately every 3 weeks. Individual panicles (the flowering parts of barley) were covered with the paper bags before they flowered to inhibit cross pollination.

Seeds obtained from the F_1 plants (F_2 hybrids) were used to generate an A × B F_2 population which was used as the donors of spikes, with anthers for anther culture studies (Figure 3.3). For the continuity of the donor plants, F_2 hybrid seeds were planted at different time intervals.

Figure 3.1. A × B F_1 hybrid seeds; (A) 3 days after, and (B) 7 days after sowing.

Figure 3.2. F_1 seedlings in the plant growth chamber maintaining vernalization conditions.

Figure 3.3. Image of donor F_2 plants used in anther culture applications.

3.1.4 Generation of A × B DHs using anther culture technique

3.1.4.1 Harvesting barley spikes

The mid-late uninucleate stage is known to be the best stage of microspore development for androgenesis in barley (Doruk et al., 2020). Determining the suitable stage for anther culture is of utmost critical importance to succeed in obtaining DHs. In this study, tillers with donor spikes were obtained by cutting between the second and third nodal areas, which were about 25 cm in length, when the flag leaves were 6-7 cm away from the second leaves (Figure 3.4) (Ohnoutkova et al., 2019). The cut tillers were taken into a beaker filled with pure water and sealed with a transparent bag to prevent moisture loss (Çetin-Özkan, 2017). Then they were kept in a refrigerator at 4°C for 3-4 days (Ohnoutkova et al., 2019).

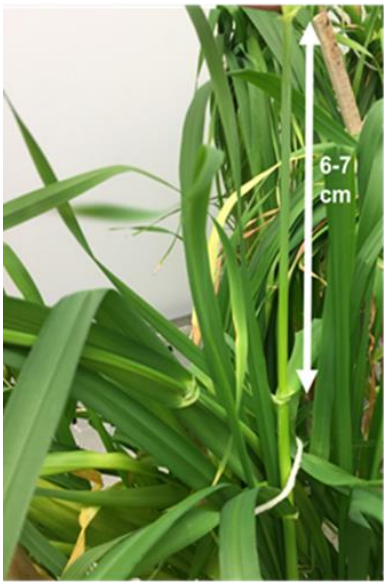

Figure 3.4. Donor tillers (or shoots) used for tissue culture study.

3.1.4.2 Isolation of anthers

Following the determination of the spike uptake time, the harvested spikes were sterilized with 70% ethanol under sterile conditions in a laminar flow cabinet. All

tissue culture applications were accomplished under sterile conditions. Anthers taken from proximal and distal spikelets of each harvested spike were removed due to possible asynchronization of the developmental stages of the pollen (Jacquard et al., 2006). Anthers were isolated with the help of a forceps from the spikelets in the middle part of the spikes taken as 25 anthers per spike (Figure 3.5).

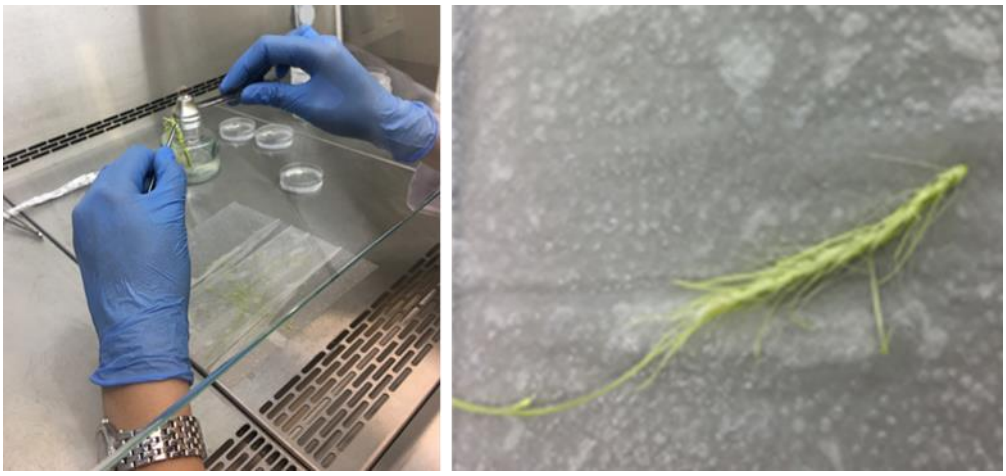

Figure 3.5. Isolation of anthers from the spikes under sterile conditions.

3.1.4.3 Pretreatment of anthers

In barley anther culture studies, it has been suggested that stress applications (pretreatment) inhibit the gametophytic pathway, and trigger pollen embryogenesis in the microspores (Cistué et al., 1998). In this study, anthers taken from the sterilized spikes were pretreated before inducing embryogenic callus. Cold applications and unmetabolizable sugar usage (such as mannitol) are examples of anther pretreatment (Çetin-Özkan, 2017). Based on these, the effects of three different pretreatment methods were evaluated in terms of triggering the formation of embryoids and green plants (Cistué et al., 2003; Kasha et al., 2003). These pretreatments involved transferring the anthers to a medium of 0.7 M mannitol (Cistué et al., 1994) or 1.0 M mannitol (Broughton et al., 2014) following the cold application to donor tillers (Ohnoutkova et al., 2019). In the first pretreatment group, the anthers were transferred onto 0.7 M (127.51 g/L) mannitol medium (with 15 g/L agar and 5.9 g/L

$CaCl_2.2H_2O$) which was poured into 6 cm diameter Petri dishes. These anthers were left in the dark at 24°C for 4 days. In the second pretreatment group, the anthers were placed onto 1.0 M (182 g/L) solidified mannitol medium using the same amount of agar and $CaCl_2.2H_2O$ by incubation under the same conditions as 0.7 M mannitol pretreatment. Also, the effect of cold pretreatment on anthers (Abdollahi et al., 2015; Wojnarowiez et al., 2004) was tested by transferring anthers into 1.0 M solidified mannitol medium and incubating in the dark at 4°C for 4 days. Then the anthers were transferred to "induction" medium, and subsequently induced calli were transferred to regeneration media developed by Broughton et al. (2014) (Table 3.1). The callus induction ratio was calculated based on the number of responding calli per 100 anthers; whereas the ratio of green plants was based on the number of calli that produce green shoots. The statistical evaluations for all parameters have been conducted using the JMP software (SAS Institute Japan Inc., Tokyo, Japan). Variance analysis has been performed with the obtained average values, and the significance of the differences has been determined according to the F-test. The Duncan test has been conducted for the grouping of differences in averages. The results were based on 100 anthers (6 repetitions) per treatment.

Table 3.1 Media for anther pretreatment trial derived from Broughton et al., (2014).

Components	Mannitol Pretreatment Medium (g/L)	Callus Formation (Induction) Medium (mg/L)	Regeneration Medium (mg/L)
Macro and Micro Salts			
KNO_3	-	1900.0	1900.0
NH_4NO_3	-	165.0	1650.0
$CaCl_2.2H_2O$	5.9	440.0	440.0
$Ca(NO_3)4H_2O$	-	-	-
$MgSO_4.7H_2O$	-	370.0	370.0
KH_2PO_4	-	170.0	170.0
KCl		-	-

"Table 3.1 (Cont'd)"

$MnSO_4.H_2O$	-	22.3	22.3
$ZnSO_4.7H_2O$	-	8.6	8.6
H_3BO_3	-	6.2	6.2
KI	-	0.83	0.83
$Na_2Mo_4.2H_2O$	-	0.25	0.25
$CoCl_2.6H_2O$	-	0.025	0.025
$CuSO_4.5H_2O$	-	0.025	0.025
Iron Source			
$FeNa_2EDTA$	-	40.0	-
$Na_2EDTA2H_2O$	-	-	37.26
$FeSO_4.7H_2O$	-	-	27.80
Other Components			
Glutamine	-	750.0	-
Myo-inositol	-	100.0	100.0
Glycine	-	-	2.0
Nicotinic acid	-	-	0.5
Pyridoxine HCl	-	-	0.5
Thiamine HCl	-	0.4	0.1
IAA	-	1.0	-
BAP	-	1.0	-
Maltose	-	100.0 g/L	-
Sucrose	-	-	20.0 g/L
Mannitol	127.51 or 182.0	-	-
Agar	15.0	10.0 g/L	-
Phytagel	-	-	3.0 g/L
pH	Not Adjusted	5.8	5.8

3.1.4.4 Anther transfer into callus formation and regeneration media

Both the induction and regeneration media were prepared at twice (2x) the necessary concentration (Ohnoutkova et al., 2019). The pH of the medium was adjusted to 5.8, and the media were sterilized through filtration by vacuum pump using a 0.22 μm cellulose acetate membrane filter assembled in a vacuum filter holder which was sterilized before usage. Phytagel or agar was used as a solidifying agent in the culture media, and these solidifiers were autoclaved at a concentration of 2x at 121°C for 20 minutes. The other media components, prepared at 2x concentrations, were mixed with 2x phytagel and/or agar in a sterile environment at a 1:1 ratio. The prepared media were then poured into 6 cm Petri dishes and stored in the dark at room temperature (RT) until usage. They were used within 4 weeks.

Pretreated anthers were transferred to medium triggering callus formation and incubated at 24°C in the dark for nearly 4 weeks to stimulate the formation of calli with embryogenic morphology. Then, calli (around 2 mm) were transferred into the regeneration medium for the encouragement of green plants/rooted green plants by culturing calli in the plant growth chamber with a temperature at 24°C for 16 h of light photoperiod. Following shoot regeneration within 2 weeks, the growing green plants were transferred from Petri dishes into Magenta GA-7 plant tissue culture boxes with polypropylene lids (Thomas Scientific, USA) and incubated for 2-3 weeks. Plantlets with underdeveloped roots and shoots were moved to a new regeneration medium for sub-culturing.

Two different combinations of callus formation and regeneration media were tested for their efficiencies. The components of these media (the first and second trial groups) are shown in Table 3.2 and Table 3.3. In the first trial group, the callus formation and regeneration medium derived from Ohnoutkova et al., (2019) were used. In the first trial group, the callus formation medium was designated as “sinduction”, and the regeneration medium was named “SR1”. Callus formation medium developed by Broughton et al., (2014) and the regeneration medium developed by Picard & De Buyser, (1973) were used in the second trial group as the

medium combination. These media were coded as “induction” and “R9”, respectively. To compare the effects of two media combinations, the anthers taken from the same spike were placed into callus formation media in an equal amount and observed for inducing embryogenic calli. Then the shoot and root formation were compared for two different regeneration media. Due to the high percentage of spontaneous chromosome doubling (about 70% of the plants) in barley (Broughton, 1999), no further chemical is used such as colchicine to obtain DHs from haploids. The callus induction ratio was calculated by counting the number of responsive calli for every 100 anthers, while the ratio of green plants was determined by the number of calli that yielded green plants. The ratio of rooted green plants represented green plants rooted out of all green shoots, while the DH ratio meant fertile and homozygous immortal lines over all rooted green plants produced through the doubled haploidy technique. All parameters underwent statistical analysis utilizing the JMP software (SAS Institute Japan Inc., Tokyo, Japan). This analysis included variance calculations using average values, and the significance of differences was assessed using the F-test. The Duncan test was applied to categorize the variations in average values. The results were based on 100 anthers (6 repetitions) per trial.

Table 3.2 Media for anther pretreatment, callus formation, and regeneration for the first trial group derived from Ohnoutkova et al., (2019).

Components	**Mannitol Pretreatment Medium (g/L)**	**Callus Formation (Sinduction) Medium (mg/L)**	**Regeneration (SR1) Medium (mg/L)**
Macro and Micro Salts			
CHU (N_6)	-	4.0 g/L	-
KNO_3	-	-	1000.0
NH_4NO_3	-	-	200.0
$CaCl_2.2H_2O$	5.9	-	-
$Ca(NO_3)_2.4H_2O$	-	-	100.0

"Table 3.2 (Cont'd)"

$MgSO_4.7H_2O$	-	-	200.0
KCl	-	-	40.0
KH_2PO_4	-	-	300.0
$MnSO_4.H_2O$	-	-	6.0
$ZnSO_4.7H_2O$	-	-	3.0
H_3BO_3	-	-	3.0
KI	-	-	0.8
$CuSO_4.5H_2O$	-	2.5	2.5
Iron Source			
$Na_2EDTA.2H_2O$	-	-	37.3
$FeSO_4.7H_2O$	-	-	27.8
Other Components			
Casein Hydrolysate	-	550.0	-
Glutamine	-	146.0	-
Myo-inositol	-	80.0	80.0
Glycine	-	3.0	3.0
Nicotinic acid	-	2.0	0.5
Pyridoxine HCl	-	2.0	0.5
Thiamine HCl	-	2.0	2.0
Biotin	-	2.0	-
2,4-D	-	2.0	-
Kinetin	-	0.5	1
NAA	-	0.5	0.5
Maltose	-	80 g/L	50 g/L
Mannitol	182.0	-	-
Agar	15.0	-	-
Phytagel	-	3.5 g/L	3 g/L
pH	Not adjusted	5.8	5.8

Table 3.3 Media for anther pretreatment, callus formation, and regeneration for the second trial group derived from Broughton et al., (2014) and Picard & De Buyser, (1973).

Components	**Mannitol Pretreatment Medium (g/L)**	**Callus Formation (Induction) Medium (mg/L)**	**Regeneration (R9) Medium (mg/L)**
Macro and Micro Salts			
KNO_3	-	1900.0	1000.0
NH_4NO_3	-	165.0	1000.0
$CaCl_2.2H_2O$	5.9	440.0	-
$Ca(NO_3)4H_2O$	-	-	500.0
$MgSO_4.7H_2O$	-	370.0	71.5
KH_2PO_4	-	170.0	300.0
KCl		-	65.0
$MnSO_4.H_2O$	-	22.3	4.9
$ZnSO_4.7H_2O$	-	8.6	2.7
H_3BO_3	-	6.2	1.6
KI	-	0.83	0.75
$Na_2Mo_4.2H_2O$	-	0.25	0.2
$CoCl_2.6H_2O$	-	0.025	0.05
$CuSO_4.5H_2O$	-	0.025	0.076
Iron Source			
$FeNa_2EDTA$	-	40.0	-
$Na_2EDTA2H_2O$	-	-	22.9
$FeSO_4.7H_2O$	-	-	17.1
Other Components			
Glutamine	-	750.0	-
Myo-inositol	-	100.0	100
Glycine	-	-	2

"Table 3.3 (Cont'd)"

Nicotinic acid	-	-	5
Pyridoxine HCl	-	-	5
Thiamine HCl	-	0.4	1
IAA	-	1.0	1
BAP	-	1.0	-
Maltose	-	100.0 g/L	-
Sucrose	-	-	20 g/L
Mannitol	182.0	-	-
Agar	15.0	10.0 g/L	5.8 g/L
pH	Not Adjusted	5.8	5.8

3.1.4.5 Vernalization, ploidy analysis and cultivation of DH plants

Once the plantlets obtained from anther culture produced at least 2-3 leaves, they were transferred into the plant growth chambers for vernalization (6°C, 8 h of light photoperiod) and kept under these conditions for 4-6 weeks. Then the ploidy levels of the plantlets were examined using Sysmex Ploidy Analyzer. To do this, 1 cm leaf samples were cut into small pieces and exposed to 0.4 ml Cysta UV ploidy (Sysmex) extraction buffer. Then, 1.6 ml of DAPI was added and the extract solution was filtered using a 30 μm nylon filter into a cuvette (Fayos et al., 2015). Each cuvette was incubated for 2 minutes in the dark. DH plants that were confirmed using ploidy analyzer were cleaned from agar and transferred to pots containing soil compost. To avoid loss of moisture, plants were covered with a plastic cover for 3 a days. Plants were grown in greenhouse or plant growth rooms in FCCRI with a temperature of 24°C with 80% relative humidity for 16 h of light photoperiod. Two weeks later, they were transferred into the large pots. They were irrigated at regular

intervals, and fungicide, insecticide, and fertilizer were applied once every three weeks until harvesting time.

3.1.4.6 Harvesting and threshing spikes of DHs and seed propagation

Spikes of DH lines were harvested and put into a separate paper bag for each line. Threshing from the spikes was performed, and all the seeds were counted for the population of individuals. DH lines with enough seeds (277 lines) were chosen for the phenotyping and genotyping analyses. The seeds of these lines were propagated as a precaution to the setbacks that might be encountered.

3.2 Phenotypical characterization of A × B DH population for *Pyrenophora teres* f. *teres*

The methodology described by Çelik Oğuz & Karakaya, (2017) and Yazıcı et al., (2015) was utilized for the phenotypical characterization of the plants at the seedling stage. Pots containing soil, sand, and organic matter (60:20:20, v/v/v, respectively) with a diameter of 7 cm and disease testing boxes were used for phenotyping the plants. Plants were grown in three replicates for each DH line in the greenhouse at Ankara University, Faculty of Agriculture, Plant Protection Department. In addition, barley cultivars Avcı 2002 and Bülbül 89 were also planted. A virulent isolate of *Pyrenophora teres* f. *teres* obtained from Sivas (GPS18) was used in the phenotyping studies. The required inoculum was derived from a 10-day-old single-spore isolate cultivated on Potato Dextrose Agar (PDA), maintained at 16°C-23±2°C with a 14 h light photoperiod. Mycelia were harvested from 10-day-old colonies by scraping from Petri dishes with a brush and washing with water to filter through a double layer of cheesecloth. Then mycelium fragments were adjusted to the concentration of 1.5-2.0×10^5 in mL (mycelium parts/ml) using a Thoma slide under the microscope (Douiyssi et al., 1998). Then a drop of Tween® 20 was added for each 100 mL inoculum suspension (Aktaş, 1995). Seedlings were inoculated in a 2.5 leaf period

at the Z12-13 stage (Zadoks et al., 1974) by spraying the inoculum suspension using a hand sprayer. After inoculation, the plants were placed into the disease testing boxes (moisture chamber) covered with nylon in the humidity circle for 72 h. Greenhouse conditions were adjusted into 18-23±2°C temperature and 14/10 h of light/dark period. The nylon covers were removed at the end of 72 hours, and the chambers were ventilated. The plants were kept under the same conditions for another 48 hours. Seven days after the inoculation, the severity of the disease was determined by using the scale described by Tekauz, (1985) (Figure 3.6). The abbreviations used in this scale were "R" for resistant (1), "R-MR" for resistant-moderately resistant (2), "MR" for moderately resistant (3), "MR-MS" for moderately resistant-moderately susceptible (4), "MR-MS" for moderately resistant-moderately susceptible (5), "MR-MS" for moderately resistant-moderately susceptible (6), "MS" for moderately susceptible (7), "MS-S" for moderately susceptible-susceptible (8), "S" for susceptible (9) and "VS" for very susceptible (10) genotypes.

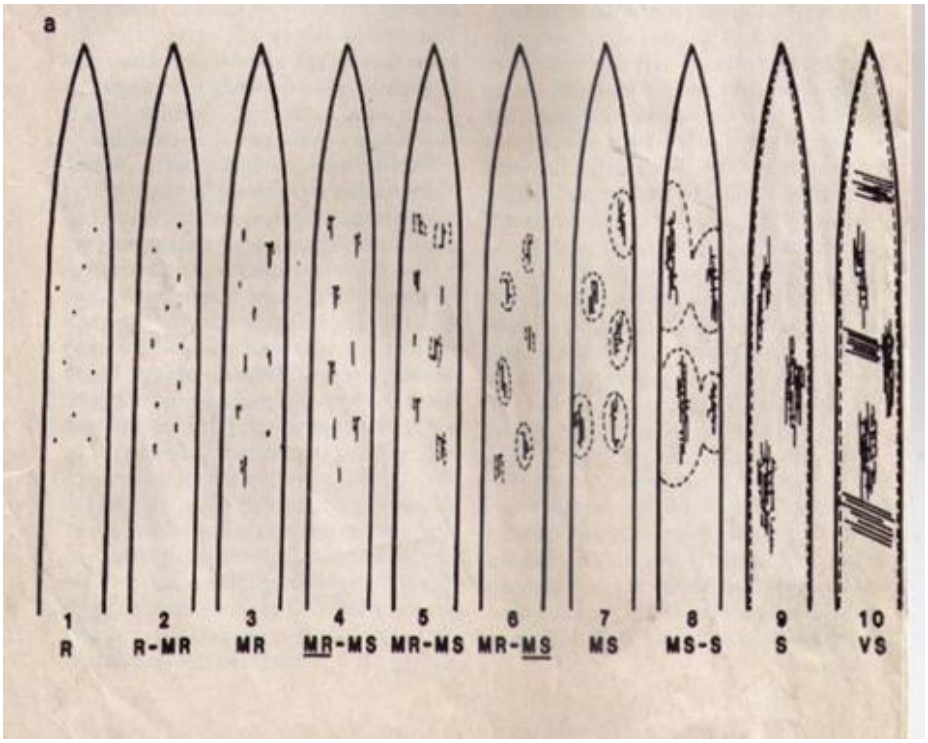

Figure 3.6. The scale used to evaluate NTNB disease in barley caused by *Pyrenophora teres* f. *teres* (Tekauz, 1985).

3.3 Genotypical characterization of A × B DH mapping population

3.3.1 DNA isolation

CTAB-based protocols (Murray & Thompson, 1980) have been successfully used for DNA extraction for GBS purposes (Abed et al., 2019). In this study, DNA samples of the DH population and the parental genotypes were extracted using the CTAB method modified from the protocols described by Allen et al., (2006) and DArT PL (Canberra, NSW, Australia) (www.diversityarrays.com). For every sample, 0.2 g of leaves were ground into a fine powder using sticks and liquid nitrogen. The extraction solutions were formulated following the protocol outlined by the company, DArT PL (www.diversityarrays.com). To make a 30 ml working buffer solution for 30 samples, first 12.5 ml extraction buffer stock (0.7975 g sorbitol, 1.25 mL 1 M Tris-HCl (pH: 8.0), 0.125 mL 0.5 M EDTA (pH: 8.0) and 11.125 mL distilled water) and 12.5 ml lysis buffer stock (0.25 g CTAB, 2.5 mL 1 M Tris-HCl (pH: 8.0), 1.25 mL 0.5 M EDTA (pH: 8.0), 5 mL 5 M NaCl, 3.75 mL distilled water) were prepared. Then, 0.15 g sodium metabisulfite and 0.6 g PVP-40 were added to the 12.5 ml extraction buffer stock. After dissolving them, 12.5 ml lysis buffer stock and 5 ml sarcosyl stock (5% (w/v)) were added to the extraction buffer stock. Freshly prepared working buffer solution was kept at 65ºC and mixed before use. The subsequent steps for DNA extraction were adapted from the procedures outlined by Allen et al., (2006). The working buffer solution in 1 ml volume was added to leaf powder in each 2 mL tube. To mix the samples with the solution, each tube was vortexed for 10 s. Then, tubes were incubated at 65ºC for 1 h by inverting once in 10 minutes. Following incubation, they were centrifuged at 13,500 g (RT) for 10 minutes. Supernatants were taken into new 2 ml tubes, and 1 mL of phenol: chloroform: isoamyl alcohol (25 v: 24 v: 1 v) solution was added to them. The tubes were gently mixed by inverting for 30 minutes. They were centrifuged at 13,500 g (RT) for 10 minutes and water phases were transferred into new tubes. After adding 800 µL of cold isopropanol (-20ºC) to the tubes, they were

gently inverted for 10 s. The tubes were kept at RT for 10 minutes. Following centrifugation at 10,000 g for 30 minutes, supernatant parts were carefully discarded, taking care not to lose the DNA pellets. DNA pellets were dissolved in 250 µL of 1x TE solution, and 2.5 µL of RNAse (10 mg/ml) was added to the tubes. The tubes were incubated at 37ºC for 30 minutes. Then, 25 µL of 3 M NaAc solution was added to them. Immediately afterward, 600 µL of cold absolute ethanol (99% v/v, -20°C) was added to the tubes, followed by inverting 5-10 times. After incubation at -20ºC for 20 minutes, they were centrifuged at 13,500 g (RT) for 10 minutes. Following the removal of supernatants, the samples were washed with 500 µL of 70% v/v cold ethanol (-20ºC). After centrifugation at 13,500 g (RT) for 10 minutes and removal of supernatant parts, the DNA pellets were dried overnight. The next day, DNA samples were dissolved in 100 µL of molecular-grade water. DNA samples were permitted to dissolve overnight at +4ºC and subsequently stored at -20ºC.

3.3.2 Quantification and qualification of DNA samples

DNA qualities and quantities of all samples were checked on 1% agarose gel using the NanoDrop 2000 UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). To make 1% agarose gel, 2.5 g agarose was dissolved in 250 ml 1xTris Borate EDTA (1xTBE) by microwaving for 2-3 min. After cooling it down for a few minutes, the agarose solution was poured into a midi gel tray with the well comb. To let the gel completely solidify, it was held at RT for 30 minutes. Then the agarose gel was placed into the gel box which was filled with 1xTBE buffer. To compare the DNA concentrations of the samples with a known DNA concentration (e.g. 50 ng DNA), 5 µL of 10 ng/uL lambda DNA (already mixed with loading dye) was loaded into one of the gel lanes. Then, 1 µL of the ladder (1 kb, 0.5 µg/µL) mixed with 1 µL of 6x loading dye and 4 µL of water was loaded into one of the lanes of each gel. The rest of the gel lanes were loaded with the DNA samples. To investigate the quality/quantity of the samples, 2 µL of DNA sample solution was mixed with 2 µL of 6x loading dye and 8 µL water. The gel was first run at 60 V for

20 min (until the samples were out of the wells), and then at 80 V for 1.5 h. To visualize the DNA samples, 600 µL Ethidium Bromide (Et-Br) (500 mg/mL) was dissolved in 600 mL water, and the gel was held in this solution for 30-45 min. Then the EtBr-stained gel was visualized by using a VILBER Quantum-ST4 1100/26MX Imaging Cabinet. To measure DNA quantity and quality using the NanoDrop, the reading was performed at 260/280 and 230/260 nm absorbance ratios. Samples of 1 µl volume were measured using 1 µl molecular grade water as the blank. The quantification of some of the DNA samples was also checked using the Qubit dsDNA HS assay kits (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The final DNA concentrations of the samples were set at 50 ng/µL.

3.3.3 DArT-seq genotyping

Genotyping of the A × B DH population and processing of the raw data was outsourced by submitting DNAs of 277 DH lines and the parental genotypes to DArT PL, Canberra, NSW, Australia. Genotyping data was generated using the DArT-seq 1.0 platform with medium density, which is a 1.2 million read assay as described on the company's website (www.diversityarrays.com). IBSC Barley Morex genome assembly (v2.0) (Monat et al., 2019) was used as the reference sequence for aligning the sequence reads and detecting the definite positions of the markers. Processing of the raw genotypic data for analyzing quality control parameters such as call rate and the polymorphic information content (PIC) was done using the DArT propriety pipeline (DArT P/L, Canberra, NSW, Australia). Processed data with identified SNP calls were assigned based on their genotype: "0" for the homozygote of the reference allele, "1" for the homozygote of the SNP allele, and "2" for the heterozygote, indicating the presence of both the reference and SNP alleles (Edet et al., 2018).

3.4 *In silico* analyses

3.4.1 Linkage map construction

Processed DArT-seq-derived SNP data was used after filtering for further analyses. Various filtering stages were applied to the raw SNPs. First, the SNPs with less than 70% call rate (more than 30% missing), showing segregation distortion, and containing 5% or more heterozygosity were eliminated. Second, the parental consensus on replications was also examined for both parents. Markers that were not identical in parental replications were eliminated. Third, SNPs with no position or positioned within 'unknown' chromosomes were also eliminated. Subsequently, any DH individual and SNP with more than 3% missing data were eliminated. The filtered SNPs were used to construct a linkage map of the A × B DH population using MapDisto 2.1.8.7. The parameters for linkage groups were first selected as a minimum logarithm of odds (LOD_{min}) of 3.0 and linkages with a recombination frequency smaller than 0.3 (r_{max} of 0.3). Subsequently the LODmin value was increased to 12 to separate markers into the seven respective chromosomes of barley. A linkage map of the A × B DH population was constructed using the Kosambi mapping function (Kosambi, 1943) to calculate marker order and genetic distances between the markers. MapDisto was used to prepare the graphical representation of LGs.

3.4.2 QTL mapping

QTL analysis was performed using the QGene program (version 4.4.0) for seedling stage resistance against *Ptt* isolate as the trait of interest. Phenotypic data was tested for normality using the Shapiro-Wilk normality test ($p < 0.05$) in QGene. QTL analyses were tested for the scanning step of 2 cM and 0.1 cM, in order. Then the QTLs were detected using CIM. Co-factors for QTL analysis were selected using default parameters. A permutation test for each QTL was performed using 1,000

permutations to determine the genome-wide LOD significance thresholds and threshold for each QTL in each LG. A confidence interval of 95% and 99% ($\alpha = 0.01$ and $\alpha = 0.05$) for QTL detection (Lynch & Walsh, 1998) was reported. A graphical representation of QTLs was prepared using ggplot2 3.4.2 within R 4.3.0. Markers' positions were compared to collapsed *Ptt* loci from the literature. The significance of neighboring markers and their physical distances from known loci were used to determine the novelty of a genetic marker (Clare et al., 2021). According to this, a significant marker was declared as the novel (i) if the closest neighboring marker, which is closely related to previously reported loci, is not significant; or (ii) if the physical distance to the nearest relevant locus is more than 10 Mbp, and there is no closer marker.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Generation of the A × B DH mapping population

4.1.1 Pretreatment of anthers

Mannitol, as a pretreatment agent, plays a significant role in androgenesis, with varying concentrations having different effects. Mannitol acts primarily as an osmotic agent, creating a stress environment that can induce the developmental switch from gametophytic to embryogenic pathways. The concentration of 0.7 M mannitol is often used to simulate drought-like conditions, promoting the initiation of embryogenesis in anthers. The elevated levels of stress due to usage of higher concentrations of mannitol, such as 1.0 M, can further enhance the embryogenic response in some barley varieties, while may also lead to increased cell damage in case the stress effect is too severe. Choosing the convenient mannitol concentration depends on the barley genotype for a successful anther culture in barley.

Cistué et al., (1994) demonstrated that 0.7 M mannitol provided simulating a mild stress environment for the initiation of embryogenesis. The osmotic pressure exerted by 0.7 M mannitol was sufficient to induce a developmental switch towards embryogenic pathways without causing significant cellular damage, as also supported by the work of Maraschin et al., (2005), which emphasized the importance of controlled osmotic conditions for successful microspore embryogenesis. On the other hand, 1.0 M mannitol pretreatment has the power to create a more intense osmotic stress environment. In a recent study done by Broughton et al., (2014), the increased stress levels with 1.0 M mannitol were more effective than the other pretreatment methods in certain barley genotypes, by enhancing the embryogenic response. In another study done by Çetin-Özkan, (2017) aiming to obtain DH plants

using barley anther culture technique, the highest callus induction ratio of 8% was determined for the different hybrids of Bülbül 89 cv. (with TARM 92 cv. and Akar cv.) based on the pretreatment with 0.7 M mannitol. In the study, the highest shoot formation rate of 80% was observed in the Bülbül 89 cv. × Zeynelağa cv. hybrids with this pretreatment, whereas the lowest shoot formation rate of 25% was detected in the Bülbül 89 × TARM 92 hybrids.

Cistué et al. (1998) also compared the effects of 0.7 M and 1.0 M mannitol for barley anther pretreatments. Calli induction and green plant ratios were 25% and 72%, respectively for 0.7 M mannitol pretreatment, while 28% and 73%, respectively for 1.0 M mannitol pretreatment of the same cultivar, Igri. On the other hand, these ratios were 28% and 19%, respectively for 0.7 M mannitol pretreatment, while 39% and 24%, respectively for 1.0 M mannitol for Reinette cv. For the barley cultivar Igri, using a mannitol concentration higher than 0.7 M did not result in a significant increase in the ratios of embryo and green plant induction. Therefore, a 0.7M mannitol concentration was deemed more suitable for this cultivar. In contrast, for the Reinette cv., both the number and percentage of green plants showed a significant increase when the mannitol concentration was raised from 0.7 M to 1.0 M. Their findings indicated that the most effective mannitol concentration for pretreatment varies depending on the specific genotype involved.

Cold pretreatment is a widely used technique to enhance the embryogenic response in anther cultures. Cold pretreatment has been generally used by exposing donor tillers of anthers before the isolation stage (Labbani et al., 2007; Ohnoutkova et al., 2019; Stober & Hessu, 1997). But there are also studies applying cold pretreatment of anthers after their isolation. Studies done by Abdollahi et al., (2015) and Wojnarowiez et al., (2004) have shown that exposing watermelon and barley anthers, respectively to low temperatures (around 4°C) for a period (typically four days) can significantly increase the efficiency of microspore embryogenesis. Moreover, Gu et al., (2004) indicated that cold treatment can alter the physiological state of the microspores, making them more responsive to embryogenic induction. The combined application of cold pretreatment of isolated anthers and mannitol is less

commonly documented. There could be synergistic effects, where the cold stress prepares the microspores, and the mannitol provides an additional trigger. Although the individual effects of cold pretreatment and 1.0 M mannitol are well-documented, their combined application to isolated barley anthers is not extensively explored in the available literature, needing for further investigation.

In this study, isolated anthers were taken into a mannitol-containing pretreatment medium after cold treatment of donor tillers. Since Broughton et al., (2014) reported that incorporating 5.9 g/L of $CaCl_2.2H_2O$ into 1.0 M mannitol medium enhanced the formation of embryogenic calli and green regenerants in barley anther culture, $CaCl_2.2H_2O$ was added into both 0.7 M and 1.0 mannitol medium. The results of the variance analysis for these hybrids are given in Table 4.1. The callus induction ratios varied between 25.0% and 51.6% with the highest value obtained at simultaneous 0.1 M mannitol and cold pretreatment. On the other hand, green plant ratios were 2.5% for 0.7 M mannitol pretreatment, while 7.7% for both 1 M mannitol, and simultaneous cold and 1.0 M mannitol pretreatments. There was no root formation for any of the groups using the regeneration medium developed by Broughton et al. (2014). According to the obtained results, there was a statistically significant difference at the 0.01 level among the 0.7 M and 1 M mannitol pretreatments in terms of calli and green plant numbers, as well as their ratios. However, cold pretreatment of anthers in 1.0 M mannitol medium was not significantly different than 1.0 M mannitol pretreatment, while 0.7 M mannitol was significantly less effective than the other two for inducing embryoids and green plants (Table 4.1). Although, some studies show that the high concentration of mannitol can lead to reduced viability (Rodriguez-Serrano et al., 2012), anthers in 1.0 M mannitol gave a better response than 0.7 M mannitol for A × B F_2 hybrids in our study. Therefore, 1 M mannitol pretreatment of anthers was chosen as the main protocol of the trial groups comparing the effects of different medium combinations.

Table 4.1 Effect of anther pretreatment with 0.7 M, 1.0 M, or cold+1.0 M mannitol for 4 days and subsequent androgenic responses of A × B hybrids.

Pretreatment	Calli ratio (%)	Callus no.	Green plant ratio (%)	Green plant no.
0.7 M mannitol	25.0±3.0 b	25.0±3.0 b	2.5±2.2 b	0.6±0.5 b
1.0 M mannitol	47.6±8.9 a	47.6±8.9 a	7.7±0.8 a	3.3±1.5 a
1.0 M mannitol + cold	51.6±9.2 a	51.6±9.2 a	7.7±0.8 a	4.0±1.0 a
P value	0.0023**	0.0023**	0.0037**	0.0044**

**Differences between means followed in same column by different letters are statistically significant P<0.01. SD (±).

4.1.2 Anther transfer into callus formation and regeneration media

In our study, the “sinduction” and “induction” media were prepared as described in Ohnoutkova et al., (2019) and Broughton et al., (2014), respectively. The Petri dishes having isolated anthers derived from the same spike were checked after an equal time. The “induction” medium triggered a better-structured callus formation compared to “sinduction” medium (Figure 4.1). It was observed that the calli in the “sinduction” medium were more fragile, while the callus color was brighter in the “induction” medium. Using “sinduction” and “induction” medium, the callus induction ratios were found to be 13.0% and 37.6%, respectively (Table 4.2). Thus, “induction” medium provided a higher calli induction ratio than the “sinduction” medium and the difference was statistically significant at the 0.01 level.

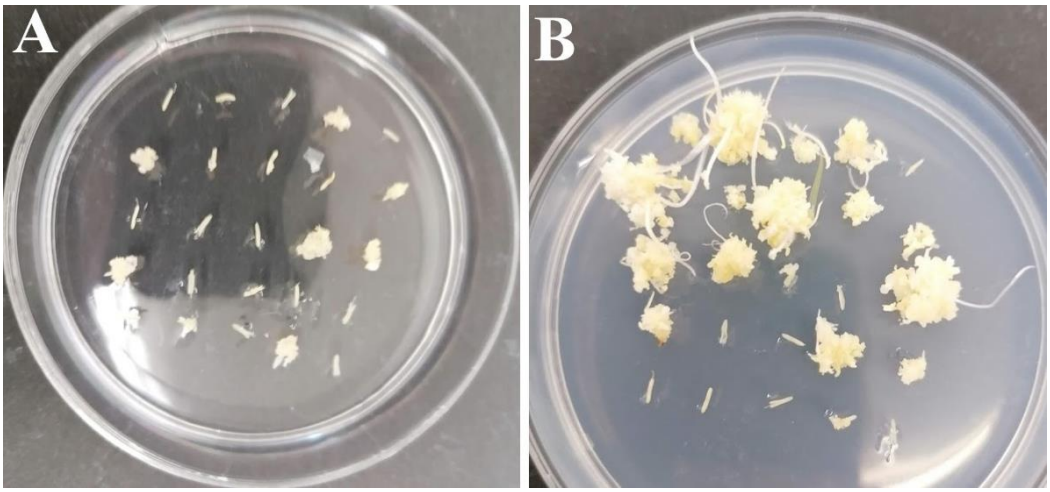

Figure 4.1. Anthers in (A) “sinduction” and (B) “induction” callus formation media following 1 M mannitol pretreatment.

Developed in the early stages of plant tissue culture, the “R9” medium developed by Picard & De Buyser, (1973) was among the pioneering regeneration media for wheat anther culture applications. The “R9” regeneration medium was used in the works of Tadesse et al., (2013, 2019) for transferring induced calli in anther culture of wheat, and the plantlets with roots were obtained successfully in about a month. To our best knowledge, there is no study using the “R9” medium in barley anther culture as the regeneration medium. In this study, we compared the effects of the regeneration

media derived from works of Ohnoutkova et al., (2019) and Picard & De Buyser, (1973). Kinetin concentration was set to 1 mg/L in "SR1", unlike Ohnoutkova et al., (2019)'s study in which the kinetin concentration was 0.5 mg/L. Unlike the originally described preparation method of "R9" (Picard & De Buyser, 1973), 5.8 g/L agar was used for barley anther culture, instead of 7 g/L agar, in this study.

When "R9" and "SR1" media were compared, the "R9" medium produced more green shoots than "SR1" (Figure 4.2). The effects of the regeneration media for the generation of DH plants are presented in Table 4.2. The green plant formation ratios were calculated as 12.5% and 24.7% in the "SR1" and "R9" medium, respectively. According to the obtained results, there was a statistically significant difference (at the 0.01 level) between the two media in terms of green plant, rooted green plant, and DH numbers, as well ratios of rooted green plants and DHs. The highest values of them were reached using the "R9" medium. On the other hand, the green plant ratio was significantly higher in the "R9" medium at the level of 0.05. Besides, no rooted green or DH plants were obtained using the "SR1" medium, whereas 93% of the green plants were rooted in the "R9" medium. The ratio of DHs obtained through spontaneous chromosome doubling from the rooted green plants was determined to be 69.1%, and an average of 6 DHs were obtained out of 100 anthers using "induction" and "R9" media, in order.

Based on the obtained results, studies were continued with the combination of the "induction" medium derived from Broughton et al., (2014) and the "R9" medium derived from Picard & De Buyser, (1973). Thus, the "induction" medium triggering a higher callus formation and the "R9" regeneration medium giving more shoots/roots were chosen for the construction of the A × B DH population (Figure 4.3).

Table 4.2 Androgenic responses of A × B hybrids from anthers with two medium combinations.

	Calli ratio (%)	Callus no.	Green plant ratio (%)	Green plant no.	Rooted green plant ratio (%)	Rooted green plant number	DH ratio (%)	DH no.
Sinduction+SR1	13.0±2.6 b	13.0±2.6 b	12.5±2.2 b	1.6±0.5 b	0.0±0.0 b	0.0±0.0 b	0.0±0.0 b	0.0±0.0 b
Induction+R9	37.6±2.5 a	37.6±2.5 a	24.7±2.4 a	9.3±1.5 a	93±6.0 a	8.6±1.1 a	69.1±6.2 a	6.0±1.0 a
P value	0.0002**	0.0002**	0.0136*	0.0075**	0.0014**	0.0059**	0.0027**	0.0091*

*Differences between means followed in same column by different letters are statistically significant P<0.05.
**Differences between means followed in same column by different letters are statistically significant P<0.01. SD (±)

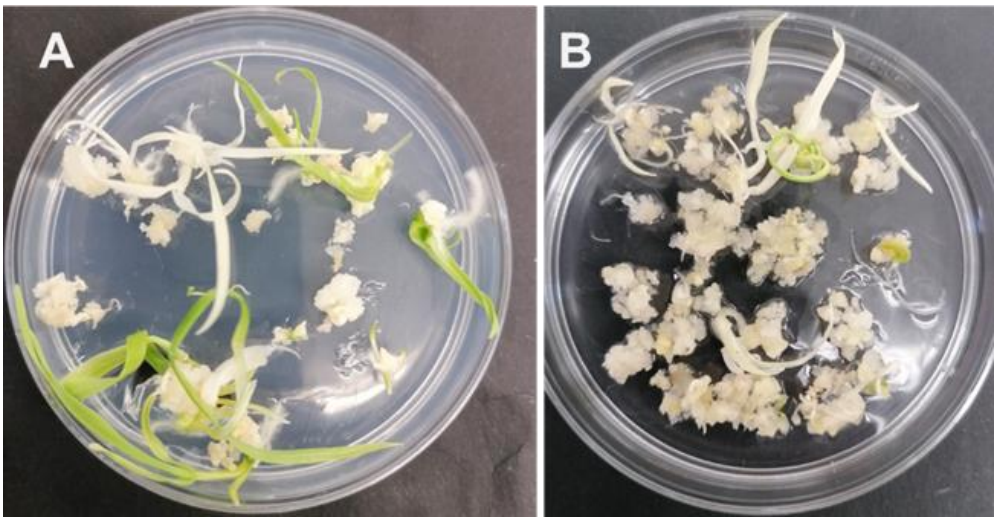

Figure 4.2. Calli transferred into regeneration media (A) “R9” medium, (B) “SR1” medium.

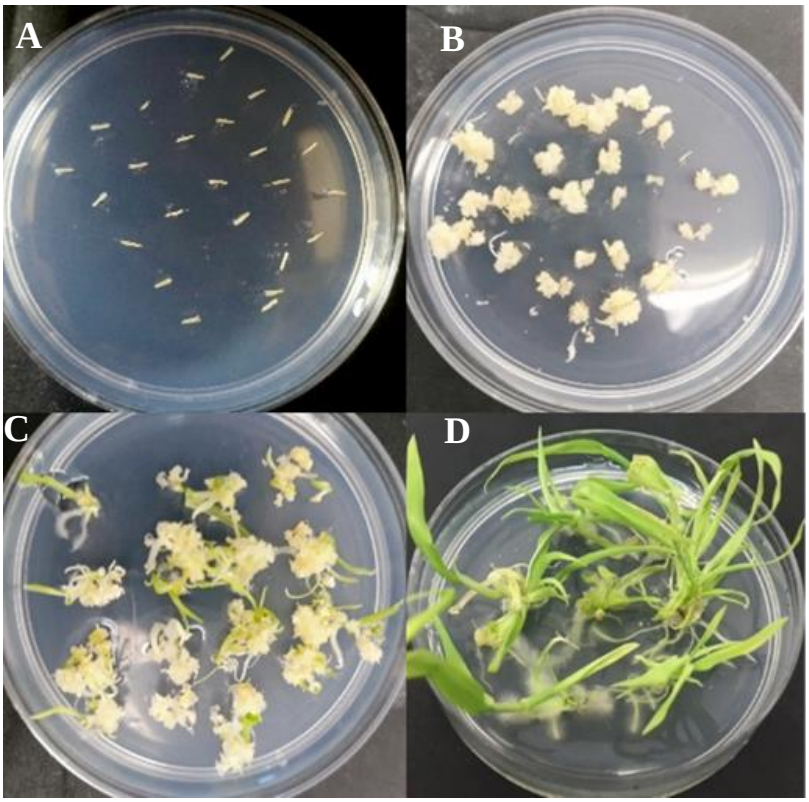

Figure 4.3. Anthers in the (A) pretreatment, (B) calli in the “induction” and (C-D) calli, and green plants in the “R9” media.

4.1.3 Cultivation of A × B DH plantlets and obtaining seeds for further analyses

Rooted green plants that were confirmed as DHs using ploidy analysis were transferred from the regeneration medium to the soil-containing pots (Figure 4.4). Out of 310 spikes, 7,750 anthers were isolated and 3,550 calli were induced. Thus 877 green plants were generated, while 815 of them were rooted and 556 of them were defined as DHs obtained from F_2 donor hybrids (Figure 4.5 and Table 4.3). The spikes of the DHs were collected when they were in the harvesting stage (Figure 4.6). Thus, 277 DH lines with enough seeds were used for genotyping and phenotyping purposes.

Figure 4.4. Transferred green plants from the R9 regeneration medium to the soil containing pots.

Figure 4.5. DH plants in the greenhouse.

Figure 4.6. Spikes of the DH plants in the greenhouse.

Table 4.3 DH plant generation responses of A × B hybrids using the optimized tissue culture protocol.

Spike number	Calli induction ratio (%)	Callus no.	Green plant ratio (%)	Green plant no.	Rooted green plant ratio (%)	Rooted green plant no.	DH ratio (%)	DH no.
310 (7,750 anthers)	45.8%	3,550	24.7%	877	92.9%	815	68.2%	556

4.2 Phenotypical characterization of A × B DH population for *Pyrenophora teres* f. *teres*

The virulent *Pyrenophora teres* f. *teres* GPS18 isolate was used in the experiments (Figure 4.7). To characterize the DH population, seeds of 277 DH lines were planted in three replicates and tested at the 2.5 leaf stage by spraying the inoculum of the *P. teres* f. *teres* isolate GPS18 (Figure 4.8 and Figure 4.9). Following inoculation, the plants in the disease boxes were covered with nylon sheets (Figure 4.10).

Figure 4.7. Preparation of the inoculum of the *Pyrenophora teres* f. *teres* isolate GPS18.

Figure 4.8. Planting five seeds into each pot, and performing testing in 3 replicates.

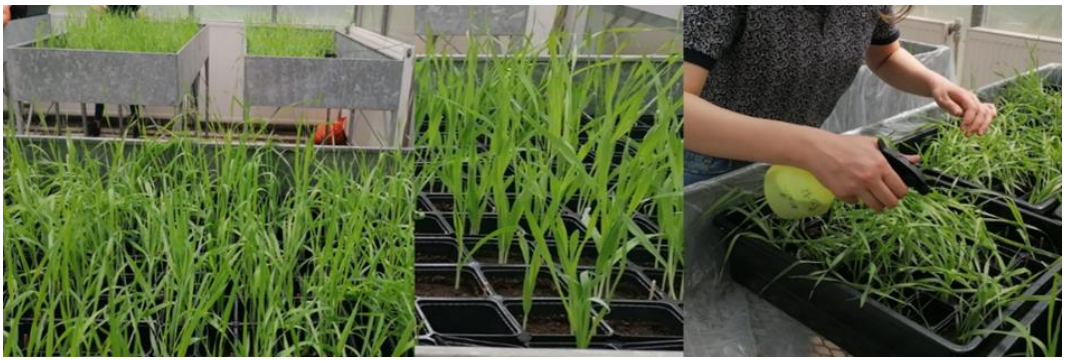

Figure 4.9. Barley plants at the 2.5 leaf stages and spray inoculation.

Figure 4.10. The plants in the disease testing boxes.

The initial signs of the disease became noticeable on the foliage of some DH lines within three to four days of inoculation. On the susceptible variety Bülbül 89, the first symptoms were observed three days after inoculation. The hallmark leaf symptoms of NTNB disease initially manifested as slim, yellow-tinged patches that progressively expanded in both width and length. Over time, leaves heavily affected

by the disease developed dark brown streaks running both lengthwise and across fashion (Figure 4.11).

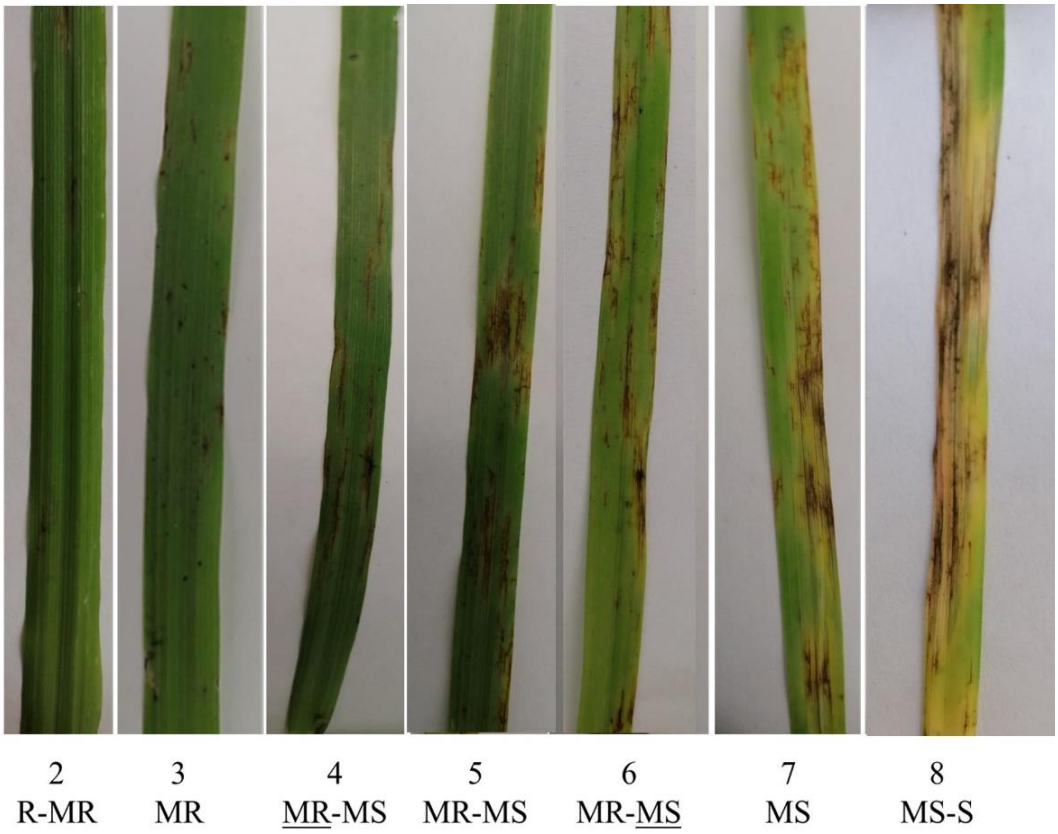

Figure 4.11. Barley leaves showing different levels of resistance to *Pyrenophora teres* f. *teres* isolate GPS18.

The severity of the disease was determined according to the symptoms on the leaves using the scale described in Tekauz, (1985). Assessments were conducted seven days after inoculation. The disease severity for each line was represented by the average scale values obtained from three replicates. GPS18 isolate of *Ptt* was used in this study as a virulent isolate, which was also used in a recent study done by Clare et al., (2021). In this study, the responses of the DH genotypes to the GPS18 isolate varied, ranging from resistant-moderately resistant to moderately susceptible-susceptible. According to the symptoms on the leaves, 3 genotypes gave R-MR (2) response, 22 genotypes showed MR (3) response and 13 genotypes showed MR-MS (4) response. On the other hand, 58 genotypes gave MR-MS (5) response, 95 genotypes gave MR-

MS (6) response, 75 genotypes gave MS (7) response, and 11 genotypes gave MS-S (8) response. Parental genotypes Avcı 2002 and Bülbül 89 exhibited a scale of 5 (MR-MS) and 8 (MS-S), respectively (Table A.1).

4.3 Genotypical characterization of A × B DH population

4.3.1 DNA isolation and quality/quantity analyses of DNA samples

DNA samples of 277 DH lines and maternal and paternal genotypes were isolated using a CTAB-based protocol. Quality and quantity determinations of the DNA samples were made using the Nanodrop device, and all samples were visualized by gel electrophoresis. Based on the NanoDrop measurements, the A260/A280 ratio needed to exceed 1.7, and the A260/A230 value to be above 2.0 to ensure the purity of DNA (Yeates et al., 1998). The absorbance ratios were found close to these values according to the NanoDrop measurements, as shown in Table 4.4 and Table B.1.

To evaluate the concentrations of the DNA samples, DNA quantities were compared to the 50-ng reference DNA, and was seen that the quantities of all the DNA samples were higher than the reference (Figure 4.12 and Figure 4.13). The DNA samples that were not suited to the quality or quantity properties were re-isolated and re-evaluated using gel electrophoresis.

Based on the outcomes of the gel electrophoresis and NanoDrop assessments, it was determined that the DNA was high quality and high quantity, and the genomic DNA was not degraded, rendering the samples suitable for the sequencing analysis (Figure 4.12 and 4.13). Measurement of some DNA samples, chosen randomly, was additionally verified through the Qubit dsDNA HS assay method to set DNA quantities into 50 ng/µl, accurately.

Table 4.4 NanoDrop analyses results of DNA samples of some DH lines.

DH line no.	DNA quantity (ng/µl)	A260/A280 ratio	A260/A230 ratio
20	375.6	1.97	2.45
21	354.4	1.96	2.46
22	461.4	1.96	2.49
23	365.6	1.98	2.41
24	273.0	1.98	2.31
25	326.0	1.95	2.42
26	372.4	1.94	2.37
27	118.2	1.95	2.43
28	278.5	1.99	2.34
29	346.1	1.98	2.37

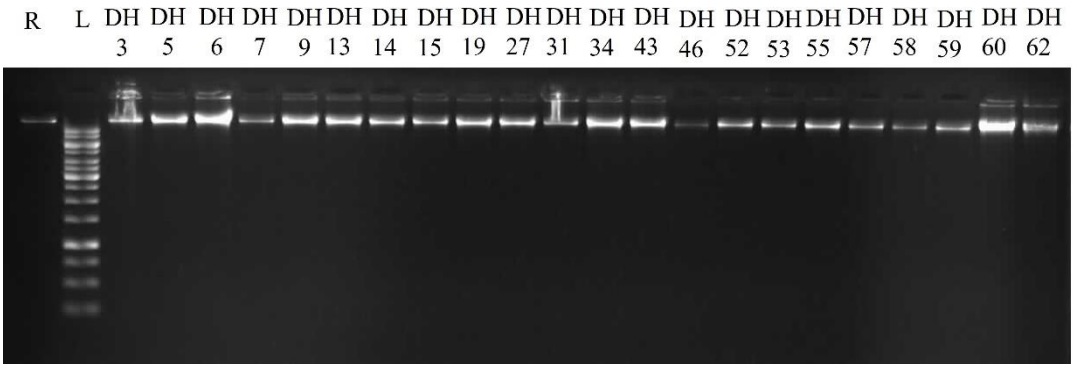

Figure 4.12. 1xTBE gel electrophoresis images of some of the DNA samples of DH lines (R: reference DNA, L: ladder, DH: DNA of DH lines).

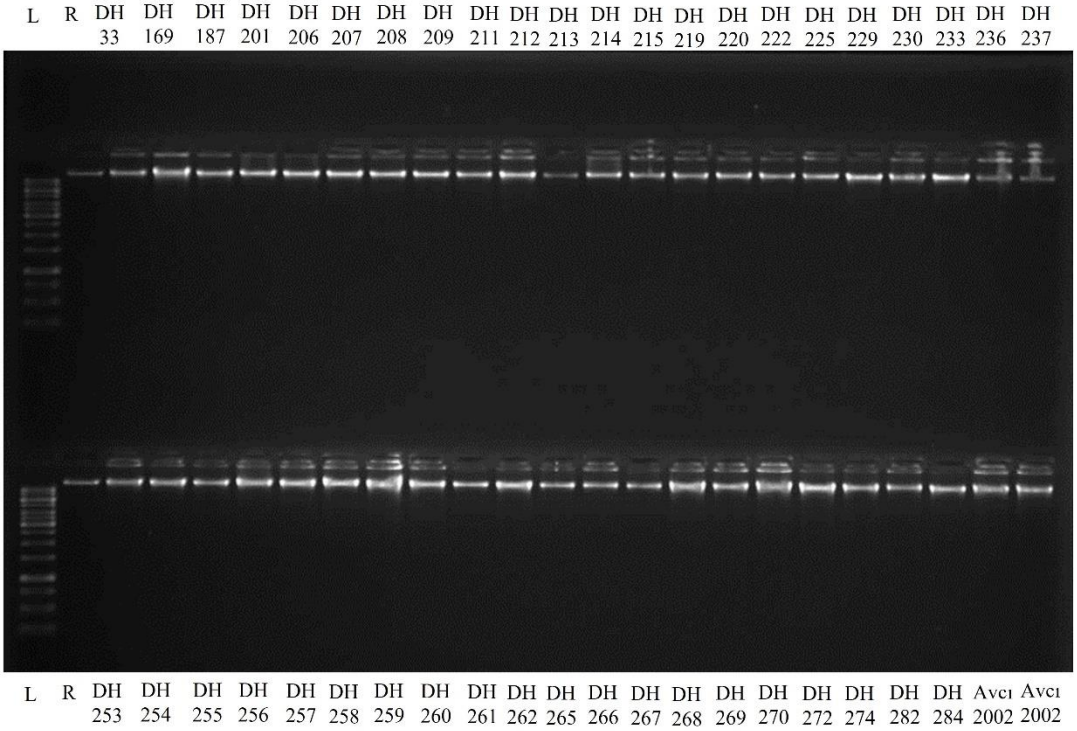

Figure 4.13. 1xTBE gel electrophoresis images of some other DNA samples of DH lines and parental line Avcı 2002 cv. (R: reference DNA, L: ladder, DH: DNA of DH lines).

4.3.2 DArT-seq genotyping

Generally, a smaller number of homozygous DH lines is enough for performing QTL analysis compared to the mapping populations constituted of non-homozygous individuals. In a recent study, Singh et al., (2018) utilized a biparental DH population named Dash/VB9104 (D/V), consisting of 92 DH lines for QTL mapping based on genotypic data obtained from "The Barley GBS 1.0 platform DArT". Since increasing the mapping population sizes can bridge the gap with markers exhibiting strong linkage and create more precise genetic maps (Piechota et al., 2019), we used 277 DH lines in this study as the biparental DH (F_2-derived) mapping population. In this study, we genotyped a biparental A × B DH population comprising 277 DH lines and its parental cultivars for obtaining a genetic map and performing QTL analysis. To do this, genomes of these accessions were sequenced using the Illumina

technology-based DArT-seq platform 1.0. The Barley GBS 1.0 platform DArT genotyping service returned 17,017 silicoDArT markers, and 9,170 SNP markers. Since the results were generally consistent between the two formats of marker types (SNPs and silicoDArTs) and phenotypic data, only codominant SNP markers were used for *in silico* analyses.

4.4 *In silico* analyses using genotypic and phenotypic data

4.4.1 Linkage map analysis

IBSC Barley Morex genome assembly (v2.0) (Monat et al., 2019) was used to define the positions of SNPs. Out of 9,170 SNPs identified from the DArT propriety pipeline; 4,512 SNPs were retained after filtering for SNPs with at least 70% call rate, segregation distortion (allele balance >70%), >5% heterozygosity, and parental consensus. A total of 2,597 SNPs remained for linkage map construction after removing SNPs with no position or with an unknown chromosome, and subsequent filtering for SNPs with more than 3% missing data. A total of 250 DH individuals from 277 DHs remained to construct a genetic linkage map.

The parameters for constructing linkage groups were first selected as default (LOD_{min}: 3 and r_{max}: 0.3). Since markers in the 2H and 5H fused, the LOD_{min} score was raised to 12. After the map was reordered, a total of 2,597 markers were organized into eight linkage groups which corresponded to the seven barley chromosomes (Table 4.5 and Figure C.1). The total map size was 1682.97 cM, and the number of positions mapped on each linkage group ranged from 81 on LG3 (3H.1) to 538 on LG2 (2H) (Table 4.5 and Figure C.1). Although a total map size of 1682.97 cM for a barley linkage map might not be exceptionally high in the realm of high-density genetic maps, it could be considered relatively dense. For example, a recent study detailed the construction of a high-density genetic map in barley using restriction-site associated DNA sequencing (Zhou et al., 2015). This map was

spanning a total length of 967.6 cM with 12,998 SNP tags mapped into seven linkage groups.

The density and the total map size can vary significantly based on the techniques used and the specific objectives of the research. In this study, the average density of markers varied, with a range of 0.82 cM/marker in LG5 (4H) to 0.45 cM/marker in LG8 (7H). The sizes of the LGs were proportional to the lengths of the barley scaffolds. LG6 (5H) was the longest at 322.76 cM, while the first part (3H.1) of the 3H in LG3 was the shortest, spanning 57.79 cM. The second part (3H.2) of the 3H was represented in the LG4, spanning 175.59 cM. Thus, the total size of the 3H in LG3 and LG4 was 233.38 cM. The 3H chromosome was split into two parts due to the missing markers (loci) within this chromosome. Thus, the DArT-seq genetic map provided the distribution of 2,597 SNP markers with an average density of 0.67 cM/marker (or 1.49 marker/cM). In a study, Rostoks et al., (2005) created a barley linkage map with a length of 1,211 cM based on 1,237 markers including RFLP, AFLP, SSR, and SNP from three DH populations and an average marker density of 1.0 marker/cM. Incorporating diverse marker types, expanding the genetic pool, utilizing advanced bioinformatics tools, and leveraging GWAS can contribute to a more detailed and accurate barley genetic map. To illustrate, in a recent study, Abed et al., (2022) developed a high-resolution consensus linkage map for barley based on GBS. This map involved 11 biparental populations comprising 3743 segregating progenies, leading to the identification of 50,875 distinct SNPs. The consensus map spanned 1050.1 cM, offering an average density of 48.4 SNP markers/cM. This map was characterized by a high resolution and uniform distribution of SNPs.

Table 4.5 Constructed linkage map of A × B DH population.

Chromosome	Linkage groups (LG)	Length (cM)	Mapped markers	Marker density (cM/marker)
1H	LG1 (1H)	196.05	254	0.77
2H	LG2 (2H)	296.17	538	0.55
3H	LG3 (3H.1)	57.79	81	0.71
3H	LG4 (3H.2)	175.59	265	0.66
4H	LG5 (4H)	237.18	289	0.82

"Table 4.5 (Cont'd)"

5H	LG6 (5H)	322.76	434	0.74
6H	LG7 (6H)	182.32	263	0.69
7H	LG8 (7H)	215.11	473	0.45
	Linkage Map	1682.97	2597	0.67

4.4.2 QTL analysis

Before QTL analysis, the normal distribution of the traits being studied is checked using a statistical program. Goh and Yap (2009) explored various normalization methods in the context of GWAS and concluded that for large sample sizes and significant genetic effects, normalization might not be necessary. Another study done by Rebaï (1997) compared the methods for regression interval mapping in QTL analysis with non-normally distributed traits. This study was an example of how non-normal distributions can be accommodated in QTL analysis with flexibility in handling various types of data distributions. These findings suggested that while normal distribution is often assumed in QTL analysis, it is not always a strict requirement, especially with the appropriate use of analysis methods tailored to the distribution of the data.

In this study, phenotypic data was assessed for normality using the Shapiro-Wilk test, with a significance level of $p < 0.05$, in the QGene software. The null hypothesis (H_0) was phenotypic data of the A × B DH mapping population was normally distributed. Since the w value was 0.937 and the p-value of the test was 0.0, the null hypothesis was rejected. The phenotypic data was accepted as not normally distributed (Figure 4.14). The Box-Cox transformation is a versatile method that includes a range of power transforms such as log transformation, which can be effective in normalizing data. Therefore, the data was transformed using log transformation in Qgene (Figure 4.15). Although the w value was reduced to 0.862, the p-value was still 0.0, meaning that the data was still not normally distributed.

Since the distribution was severely skewed and could not be normalized, the CIM-based QTL scan interval was reduced from 2.0 to 0.1 cM for a more detailed analysis.

Therefore, first, the QTL scans were performed by computing at 2 cM intervals across the entire genome once the data was accepted as normally distributed. Second, the QTL analysis was performed with a scanning step of 0.1 cM. Between QTL analyses based on the 2 cM and 0.1 cM scan interval, there were small changes in the LOD values of the SNPs within QTLs. Furthermore, the positions of the QTL flanking markers were changed, which let the QTLs narrow down. On the other hand, there were no changes in the physical/genetic positions of the SNP markers at the QTL peaks. Therefore, the QTLs were eventually detected based on a genome scan at 0.1 cM step intervals so as not to skip anything important at the small interval of a scan.

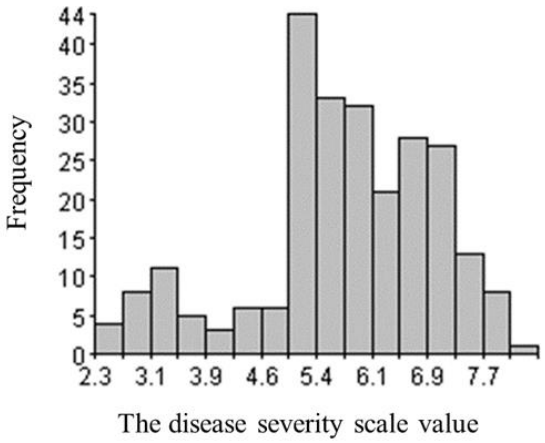

Figure 4.14. Phenotypic data underwent a normality assessment via the Shapiro-Wilk test ($p < 0.05$).

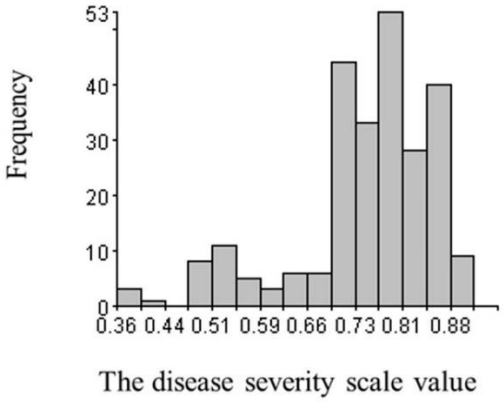

Figure 4.15. Phenotypic data underwent a normality assessment via the Shapiro-Wilk test ($p < 0.05$) after log transformation.

CIM and Single Marker Regression (SMR) are the two methods used in QTL analysis, but they differ in several key ways. CIM is more complex and generally more accurate than SMR. It uses multiple markers to control the genetic background, thereby reducing background noise and increasing the accuracy of QTL detection. CIM controls for background genetic variation by using markers outside the testing interval as covariates, while SMR analyzes one marker at a time without controlling for the genetic background. Furthermore, CIM tends to have higher statistical power for detecting QTLs compared to SMR. This is because CIM can separate the effects of linked QTLs, which might be confounded in SMR. Also, CIM is computationally more intensive than SMR, due to its more complex analysis involving multiple markers and intervals. Since CIM is better for accurate and detailed QTL mapping in well-characterized populations, the QTL analysis based on CIM was performed for this study.

Based on CIM-based QTL analysis, a total of three QTLs were revealed for *Ptt* resistance as the trait. The LOD scores of the QTL peaks were 4.757 (α0.05), 4.427 (α0.05), and 5.429 (α0.01), on the 3H, 4H, and 6H, respectively. The LOD scores which were between the LOD threshold (upper limit and lower limits) were indicated that these three QTLs were statistically significant at the levels of α0.05 and α0.01.

The QTLs gave peaks at the physical positions of the loci (SNP markers) at 3H_654,310,071, 4H_575,415,754, and 6H_19,609,460, while their genetic positions were 142, 117, and 45 cM, respectively. The SNPs flanking these QTLs were determined by checking whether the LOD scores of the closest SNPs to QTL peaks go out of the LOD threshold limits. Thus, the physical positions of the SNPs flanking the QTLs were between 3H_652,500,267 – 3H_654,337,208, 4H_569,060,926 – 4H_576,137,567 and 6H_19,490,480 – 6H_24,080,319. On the other hand, the genetic positions of SNPs flanking the QTLs were between 139 and 148 cM; 113 and 119 cM; and 40 and 52 cM on chromosomes 3H, 4H, and 6H, respectively (Table 4.6 and Figure 4.16).

Table 4.6 SNPs at the peaks of the QTLs associated with disease resistance and the SNPs flanking the QTLs on the 3H, 4H and 6H chromosomes.

	Physical positions of the SNP markers	Genetic positions of the SNP markers (cM)	CIM (LOD)	Upper limit for LOD threshold	Lower limit for LOD threshold
3H-SNP flanking the QTL	3H_652,500,267	139.0	0.061	5.429	4.204
3H-SNP at the peak of the QTL	**3H_653,276,496**	**141.0**	**4.555**	**5.429**	**4.204**
3H-SNP at the peak of the QTL	**3H_654,310,071**	**142.0**	**4.757**	**5.429**	**4.204**
3H-SNP flanking the QTL	3H_654,337,208	148.0	0.0	5.429	4.204
4H-SNP flanking the QTL	4H_569,060,926	113.0	11.383	5.429	4.204
4H-SNP at the peak of QTL	**4H_575,415,754**	**117.0**	**4.427**	**5.429**	**4.204**
4H-SNP flanking the QTL	4H_576,137,567	119.0	0.039	5.429	4.204
6H-SNP flanking the QTL	6H_19,490,480	40.0	2.605	5.429	4.204
6H-SNP at the peak of the QTL	**6H_19,609,460**	**45.0**	**5.425**	**5.429**	**4.204**
6H-SNP flanking the QTL	6H_24,080,319	52.0	0.029	5.429	4.204

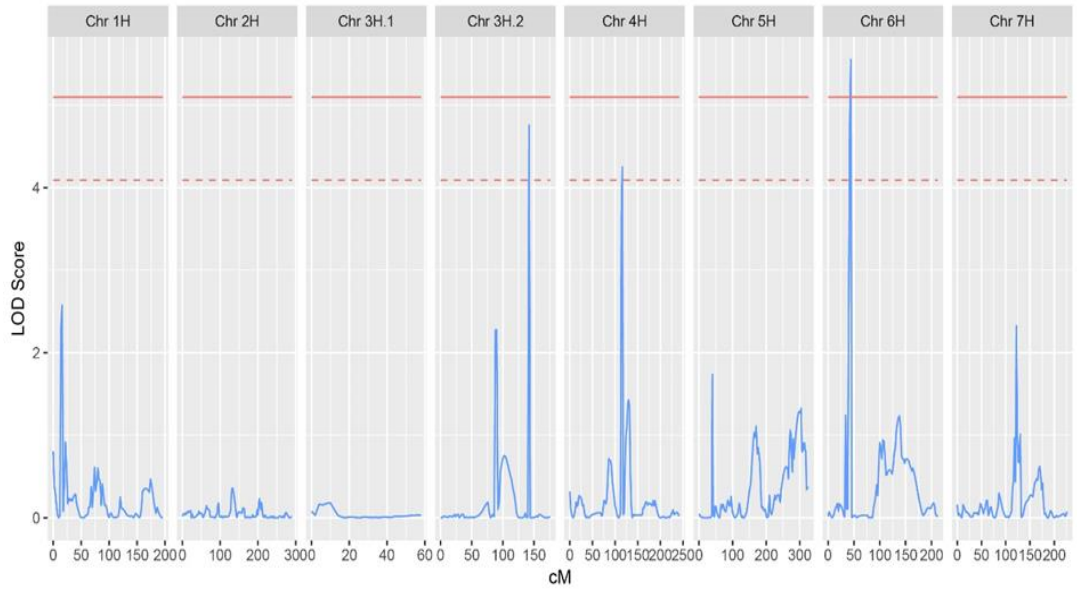

Figure 4.16. QTL analysis result showing significant QTLs (α0.01 and α0.05) between the upper and lower threshold limits (LOD threshold limits: 5.429 and 4.204).

In addition, CIM-based QTL analysis gave information about the values of R^2 showing the phenotypic variation explanation percentage by each QTL (Table 4.7). The QTL on 6H was accounted for 9.7% variation, while the other two were 8.4% and 7.5% on 3H and 4H, respectively. So that identified QTLs explained almost 26% of the total phenotypic variance.

Table 4.7 Test statistics for Composite Interval Mapping (CIM) showing the percentage of the variance explained by each QTL (R^2).

Chromosomes of the detected QTLs	Physical positions of SNP markers at the QTL peaks	Genetic positions of SNP markers at the QTL peaks (cM)	CIM (R^2) phenotypical variance explained by each QTL) (%)	CIM (LOD)
3H	3H_654,310,071	142.0	8.4	4.757
4H	4H_575,415,754	117.0	7.5	4.427
6H	6H_19,609,460	45.0	9.7	5.425

The previously reported loci and genes were compared with the identified genomic regions based on the QTL analysis of the A × B DH population by using the sequence

of the genome and the existing literature. The QTL on 3H (3H_652,500,267 – 3H_654,337,208) was potentially novel. The potentially novel QTL was approximately 25 Mbp away from the previously identified *QRptts-3HL* locus (Adhikari et al., 2019, 2020; Amezrou et al., 2018; Daba et al., 2019; Richards et al., 2017; Rozanova et al., 2019; Tamang et al., 2015) which was the nearest QTL. The *QRptts-3HL* locus, close to the telomeric region on the long arm of the 3H chromosome, was defined in a study by Richards et al., (2017) for *Ptt* resistance using 15A isolate. In that study, GWAS analysis was conducted on geographically diverse barley genotypes (957 barley lines) to detect marker-trait associations (MTAs) for resistance or susceptibility to *Ptt*. The GWAS analysis identified 78 significant MTAs at 16 unique genomic loci. Among these, 5 loci were novel, and one of them was *QRptts-3HL*. On the other hand, a QTL called *QRptta-3H-154-155* was identified on the 3H in the work of Amezrou et al., (2018), and based on the physical positions of the markers flanking the QTLs related, the *QRptta-3H-154-155* locus was covering the *QRptts-3HL* locus (Clare et al., 2020). Thus, the locus on 3H defined in our study was potentially a novel QTL since the physical distance to the nearest relevant locus was over 10 Mbp.

On the other hand, the QTL identified in this study on chromosome 4H was between the physical positions of 4H_569,060,926 – 4H_576,137,567 (Novakazi et al., 2019; Raman et al., 2003). Based on the physical positions of the markers related, the currently identified QTL on 4H might be covering the *Rpt7* locus on 4HL (Adhikari et al., 2020; Afanasenko et al., 2015; Martin et al., 2018; Raman et al., 2003; Richards et al., 2017; Tamang et al., 2015, 2019; Vatter et al., 2017; Wonneberger et al., 2017; Yun et al., 2005). There are previous synonyms of *Rpt7* locus (*QRpts4L*, *Rpt-4H-5–7*, *QRpts4*, *AL_QRptt4-1*, *Qpt.4H-3* and *QRptm-4H-58-64*) based on their positions on 4H (Clare et al., 2020). Raman et al., (2003) performed QTL analysis based on the Sloop/Halcyon DH population using SSR and AFLP markers, and defined a major gene locus, explaining 64% of the phenotypic variation. This locus was designated as *QRpts4L,* associated with NTNB resistance at the seedling stage. They also validated this locus using the F_2 population of Ant29/Halcyon (81 lines)

with the SSR markers. These markers could accurately predict 93% of the DH lines as resistant or susceptible, showing that they could be employed in breeding programs (MAS applications) to choose alleles beneficial for NTNB resistance. Then, Yun et al., (2005) performed SSR marker-based genotyping and QTL analysis of a RIL population, derived from crossing the Harrington cultivar with wild barley (*H. spontaneoum*) which is a resistance source to biotic stress factors. Thus, a QTL, designated *Rpt-4H-5–7* locus was detected explaining 10% and 9% of the phenotypic variation for resistance to NTNB in two greenhouse experiments. In a recent study, Tamang et al., (2015) used the Illumina barley iSelect array and two isolates of *Ptm* derived from New Zealand (NZKF2) and Denmark (DEN 2.6). An association mapping was conducted by these researchers on a worldwide collection, consisting of 1,480 barley accessions. A shared genetic region for the NZKF2 and DEN 2.6 isolates, previously identified as *QRpts4*, was pinpointed on the 4H, spanning from 53.67 to 59.22 cM. This region accounted for as much as 16% of the traits' variation. Within this region, 36 significant markers were identified for both isolates. However, the markers associated with NZKF2 showed the greatest significance among all MTAs examined in the research. In the same year, Afanasenko et al., (2015) detected a QTL for *Ptt* resistance by mapping a DH population derived from Zernogradsky 813/Ranniy1 hybrids, and the interval between the SNP markers related to this QTL was 52-59 cM. Afterward, the *AL_QRptt4-1* locus on 4H was identified by Wonneberger et al., (2017) at the seedling stage associated with the resistance against isolates of *Ptt*. Although this locus was not prominent in the adult stage field experiments, its LOD score implied that this locus could still play a minor role in the adult stage resistance. In a recent study, Vatter et al., (2017) conducted a nested association mapping analysis using the NAM population. The NAM population constituted 1,420 backcross inbred lines. These lines were developed from a cross between 25 diverse wild barley accessions, specifically *Hordeum vulgare* subspecies *spontaneum* and *Hordeum agriocrithon*, with the modern spring barley variety known as "Barke", belonging to *Hordeum vulgare* subspecies *vulgare*. The nested association mapping was done to identify QTL for resistance against *Ptt* by scoring

the reaction type in the field trials. The detected QTL, designated as *Qpt.4H-3*, was found to correspond to the previously detected QTL, *QRpts4*. A year later than this study, Martin et al., (2018) defined a QTL for resistance against *Ptt* in UVC8/Erica population (South African barley breeders' lines) with a LOD score of 3.6. The approximate physical position of the marker related to this locus overlapped with the *Rpt7* locus (Clare et al., 2020). In the study of Tamang et al., (2019), a RIL (Tra_67381) population was used to discover QTLs for resistance against the DEN2.6 and NZKF2 *Ptm* isolates. The discovered QTL on 4H, designated as *QRptm-4H-58-64*, was situated between SNP markers at 58.82 cM and 64.45 cM. The LOD scores for this QTL were 7.84, explaining 26% of the phenotypic variance, and 10.88 accounting for 34% of the variance against DEN2.6 and NZKF2 isolates, respectively. Overall, the *Rpt7* locus has been defined as related to the resistance against both forms of *P. teres* (Clare et al., 2020). The currently identified locus on 4H, possibly overlapping the *Rpt7* locus, seems to play a role in the seedling resistance against *Ptt* in the A × B DH population.

In our study, the QTL on chromosome 6H (6H_19,490,480 – 6H_24,080,319) likely overlapped with the *SFNB-6H-33.74* locus as reported by Amezrou et al., (2018); Burlakoti et al., (2017); Daba et al., (2019). Burlakoti et al., (2017) identified the *SFNB-6H-33.74* locus based on GWAS analysis of 376 barley breeding lines investigating loci associated with *Ptm* at the seedling stage. Chromosome 6H is known to have an important role in resistance against NB, as reported in the works of Clare et al., (2020) and Liu et al., (2011). The *QRptta-6H-35.62* locus, associated with NTNB resistance identified by Amezrou et al., (2018) was located 1.88 cM away from the *SFNB-6H-33.74* locus. According to this proximity, the researchers suggested that the *QRptta-6H-35.62* locus may share common genes with *SFNB-6H-33.74* that impart resistance to both types of NB and/or it might encompass two closely linked but separate genes responsible for resistance. In recent work, Daba et al., (2019) identified a QTL (*Qnfnb-6H.4*) on chromosome 6H for NTNB resistance, and this QTL was located approximately 2.5 Mbp distant from the QTL identified by Burlakoti et al., (2017), possibly covering the *SFNB-6H-33.74* locus. This QTL

was highly associated with NTNB resistance at the seedling stage, explaining 9% of the phenotypic effect. Hence, the currently identified locus on 6H in our study, which seems to overlap with the *SFNB-6H-33.74* locus, has been supportive of the studies emphasizing the role of the genomic regions on chromosome 6H in resistance to *Ptt*.

During the QTL analysis in our study, test statistics for CIM analysis provided identification of SNPs associated with *Ptt* resistance which were within the defined QTLs (Table 4.6). All the SNP markers in Table 4.6 were significantly associated with the trait. Thus, PCR-based KASP markers can be developed for verifying these SNPs. To develop KASP markers, these SNPs and the sequence surrounding each SNP can be used for designing the KASP markers. In Table 4.6, the SNPs shown in the pink rows were the QTL peak markers, which can be a good starting point for the validation of identified markers. Additionally, markers close to the peaks of QTLs can be chosen for marker development.

CHAPTER 5

CONCLUSION

Developing resistant cultivars by identifying resistance sources against *Ptt*, which is the causal agent of a major barley disease NTNB, is the most environmental and sustainable solution. The ability to select disease-resistant genotypes using molecular markers, independent of the pathogen and the developmental stage of the plant, could help reduce the risks of yield reduction or total loss of the crop. Barley breeding germplasm in Turkey offers a diverse pool for MAS. The research presented here lays the foundation for the first step to the development of DNA tests that can identify resistant genotypes and the development of resistant barley cultivars, eventually. To achieve this goal, a biparental barley DH population was developed based on the anther culture technique using hybrids of cultivars (Avcı 2002 and Bülbül 89), segregating for this trait. To generate the A × B DH population, donor tillers were held at 4°C for 3-4 days and the isolated anthers were placed onto 1 M mannitol medium and incubated at 4°C for 4 days. Then pretreated anthers were transferred into the "induction" medium derived from Broughton et al., (2014) and the "R9" regeneration medium derived from Picard & De Buyser, (1973) for callus formation, and shoot/root generation subsequently.

SNP markers in the DH mapping population were identified using a GBS technique designated as DART-seq, enabling the construction of a linkage map for QTL analysis. The genetic linkage map developed in this study has provided information that described genomic regions related to *Ptt* resistance, possibly overlapping with the previously reported QTLs in the literature and/or revealing potentially novel ones. QTL analysis suggested that three QTLs on 3H, 4H, and 6H have the most significant impact on NTNB resistance in the DH mapping population based on barley cultivars derived from Turkey. QTLs overlapping with the physical positions of previously identified QTLs in the literature supported the importance of resistance

genes within these genomic regions. For example, the QTL associated with *Ptt* resistance on 6H have been demonstrated in many other studies (Amezrou et al., 2018; Burlakoti et al., 2017; Daba et al., 2019; Mazinani et al., 2020). In this study, the QTL identified on chromosome 6H was likely overlapping with the previously described *SFNB-6H-33.74* locus. Meanwhile, the QTL discovered on chromosome 3H could be novel since it was approximately 25 Mbp away from the previously defined *QRptts-3HL* locus. Additionally, the QTL on chromosome 4H might coincide with the *Rpt7* locus which takes a role in both *Ptt* and *Ptm* resistance.

The disease-resistance-associated SNPs within the QTLs identified in this study are promising candidate markers. This study has paved the way for the development of a user-friendly DNA-based assay by identifying associated SNP markers for NTNB resistance as the trait of interest. Development of a KASP marker assay based on information on these SNPs may enable the rapid and reliable verification of individuals as resistant or susceptible genotypes. This will shed light on the development of a quick and cost-effective test to be used in barley resistance breeding programs.

In conclusion, the results presented here provide information on the trait-associated SNP markers which may be suitable for MAS applications related to *Ptt* resistance in barley germplasms. The future aim of the subsequent studies will be the validation of these candidate markers by developing a DNA test that can be routinely applied. Additionally, fine mapping can be performed to narrow down the identified QTLs in the future. The genetic map of the A × B DH population can also be used to map genomic regions of resistance to other fungal agents such as *Pyrenophora teres* f. *maculata* and *Rhynchosporium commune*, as well as other traits of interest segregated for these varieties. Overall, this is the first study focusing on genetic and QTL mapping using Turkish barley cultivars. We believe that this study will contribute to identifying resistance sources in barley breeding programs with the help of identified resistance-associated SNP markers for fighting against NFNB caused by *Pyrenophora teres* f. *teres*.

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APPENDICES

A. Phenotypical characterization of A × B DH population for *Pyrenophora teres* f. *teres*

Table A.1 Disease testing evaluations of A × B DH lines against *Pyrenophora teres* f. *teres*. SD (±).

Genotype	The average scale value of 3 replicates	Disease testing category
Genotype 1	6.66±0.71	**MS**
Genotype 10	5.66±0.58	**MR-MS**
Genotype 100	6.66±0.58	**MS**
Genotype 101	5.66±0.58	**MR-MS**
Genotype 102	7.33±0.58	**MS**
Genotype 103	5.66±0.58	**MR-MS**
Genotype 104	3.00±0.00	**MR**
Genotype 105	6.00±0.00	**MR-MS**
Genotype 106	5.00±0.00	**MR-MS**
Genotype 107	5.33±0.58	**MR-MS**
Genotype 108	7.66±0.58	**MS-S**
Genotype 109	5.66±0.71	**MR-MS**
Genotype 11	6.00±0.00	**MR-MS**
Genotype 110	5.00±0.00	**MR-MS**
Genotype 111	7.00±0.00	**MS**
Genotype 112	5.66±0.58	**MR-MS**
Genotype 113	7.00±0.00	**MS**
Genotype 114	6.66±0.58	**MS**
Genotype 115	6.33±0.58	**MR-MS**
Genotype 116	6.00±0.00	**MR-MS**
Genotype 117	6.00±0.00	**MR-MS**
Genotype 118	5.33±0.58	**MR-MS**
Genotype 119	6.00±0.00	**MR-MS**
Genotype 12	3.33±0.58	**MR**
Genotype 120	6.33±0.58	**MR-MS**
Genotype 121	4.66±0.71	**MR-MS**
Genotype 122	3.66±0.58	**MR-MS**

"Table A.1 (Cont'd)"

Genotype 123	7.00±0.00	**MS**
Genotype 124	5.33±0.58	**MR-MS**
Genotype 125	6.66±0.58	**MS**
Genotype 126	6.00±0.00	**MR-MS**
Genotype 127	3.33±0.58	**MR**
Genotype 128	5.00±0.00	**MR-MS**
Genotype 129	5.66±0.58	**MR-MS**
Genotype 13	6.00±0.00	**MR-MS**
Genotype 130	5.33±0.58	**MR-MS**
Genotype 131	2.33±0.58	**R-MR**
Genotype 132	6.33±0.58	**MR-MS**
Genotype 133	3.33±0.58	**MR**
Genotype 134	7.00±0.00	**MS**
Genotype 135	7.00±0.00	**MS**
Genotype 136	6.00±0.00	**MR-MS**
Genotype 137	6.00±0.00	**MR-MS**
Genotype 138	5.33±0.58	**MR-MS**
Genotype 139	7.00±0.00	**MS**
Genotype 14	6.00±0.00	**MR-MS**
Genotype 140	5.00±0.00	**MR-MS**
Genotype 141	5.66±0.58	**MR-MS**
Genotype 142	5.33±0.58	**MR-MS**
Genotype 143	5.00±0.00	**MR-MS**
Genotype 144	6.33±0.58	**MR-MS**
Genotype 145	5.33±0.58	**MR-MS**
Genotype 146	5.66±0.58	**MR-MS**
Genotype 147	6.33±0.58	**MR-MS**
Genotype 148	7.00±0.00	**MS**
Genotype 149	4.00±0.00	**MR-MS**
Genotype 15	7.33±0.58	**MS**
Genotype 150	6.00±0.00	**MR-MS**
Genotype 151	8.00±0.00	**MS-S**
Genotype 152	6.33±1.15	**MR-MS**
Genotype 153	6.33±0.58	**MR-MS**
Genotype 154	6.00±0.00	**MR-MS**
Genotype 155	5.66±0.58	**MR-MS**
Genotype 156	4.66±0.58	**MR-MS**

"Table A.1 (Cont'd)"

Genotype 157	6.00±0.00	**MR-MS**
Genotype 158	5.00±0.00	**MR-MS**
Genotype 159	7.33±0.58	**MS**
Genotype 16	7.00±0.00	**MS**
Genotype 160	6.66±0.58	**MS**
Genotype 161	3.33±0.58	**MR**
Genotype 162	6.00±0.00	**MR-MS**
Genotype 163	7.33±0.58	**MS**
Genotype 164	7.00±0.00	**MS**
Genotype 165	4.66±0.58	**MR-MS**
Genotype 166	5.66±0.58	**MR-MS**
Genotype 167	6.00±0.00	**MR-MS**
Genotype 168	6.66±0.58	**MS**
Genotype 169	5.00±0.00	**MR-MS**
Genotype 17	6.00±0.00	**MR-MS**
Genotype 170	6.00±0.00	**MR-MS**
Genotype 171	6.66±0.58	**MS**
Genotype 172	3.33±0.58	**MR**
Genotype 173	5.66±0.58	**MR-MS**
Genotype 174	5.66±0.71	**MR-MS**
Genotype 175	6.00±0.00	**MR-MS**
Genotype 176	2.33±0.58	**R-MR**
Genotype 177	6.33±1.15	**MR-MS**
Genotype 178	6.00±0.00	**MR-MS**
Genotype 179	3.00±0.00	**MR**
Genotype 18	3.00±0.00	**MR**
Genotype 180	6.00±0.00	**MR-MS**
Genotype 181	6.00±0.00	**MR-MS**
Genotype 182	3.00±0.00	**MR**
Genotype 183	5.00±0.00	**MR-MS**
Genotype 184	3.33±0.58	**MR**
Genotype 185	4.33±0.58	**MR-MS**
Genotype 187	6.00±0.00	**MR-MS**
Genotype 188	5.33±0.58	**MR-MS**
Genotype 189	7.33±0.58	**MS**
Genotype 19	6.00±0.00	**MR-MS**
Genotype 190	5.00±0.00	**MR-MS**

"Table A.1 (Cont'd)"

Genotype 191	5.66±0.58	**MR-MS**
Genotype 192	6.33±0.58	**MR-MS**
Genotype 193	6.00±0.00	**MR-MS**
Genotype 194	4.66±1.15	**MR-MS**
Genotype 195	3.33±0.58	**MR**
Genotype 196	5.66±0.58	**MR-MS**
Genotype 197	7.33±0.58	**MS**
Genotype 198	3.00±0.00	**MR**
Genotype 199	4.50±0.71	**MR-MS**
Genotype 2	6.33±0.58	**MR-MS**
Genotype 20	6.66±0.58	**MS**
Genotype 200	6.00±0.00	**MR-MS**
Genotype 201	6.66±0.58	**MS**
Genotype 202	5.33±0.58	**MR-MS**
Genotype 203	7.00±0.00	**MS**
Genotype 204	5.00±0.00	**MR-MS**
Genotype 205	6.66±0.58	**MS**
Genotype 206	2.66±0.58	**MR**
Genotype 207	6.00±0.00	**MR-MS**
Genotype 208	6.66±0.58	**MS**
Genotype 209	7.33±0.58	**MS**
Genotype 21	7.00±0.00	**MS**
Genotype 210	6.33±0.58	**MR-MS**
Genotype 211	5.66±0.58	**MR-MS**
Genotype 212	7.00±0.00	**MS**
Genotype 213	6.66±0.58	**MS**
Genotype 214	6.66±0.58	**MS**
Genotype 215	7.66±0.58	**MS-S**
Genotype 216	6.66±0.58	**MS**
Genotype 217	5.33±0.58	**MR-MS**
Genotype 218	7.66±0.58	**MS-S**
Genotype 219	5.66±0.58	**MR-MS**
Genotype 22	3.00±0.00	**MR**
Genotype 220	5.33±0.58	**MR-MS**
Genotype 221	5.33±0.58	**MR-MS**
Genotype 222	7.33±0.58	**MS**
Genotype 223	4.33±0.58	**MR-MS**

"Table A.1 (Cont'd)"

Genotype 224	5.66±0.58	**MR-MS**
Genotype 225	7.33±0.58	**MS**
Genotype 226	6.33±0.58	**MR-MS**
Genotype 227	5.66±0.58	**MR-MS**
Genotype 228	3.66±0.58	**MR-MS**
Genotype 229	7.00±0.00	**MS**
Genotype 23	6.66±0.58	**MS**
Genotype 230	5.33±0.58	**MR-MS**
Genotype 231	6.00±0.00	**MR-MS**
Genotype 232	5.33±0.58	**MR-MS**
Genotype 233	4.33±0.58	**MR-MS**
Genotype 234	6.33±1.15	**MR-MS**
Genotype 235	5.33±0.58	**MR-MS**
Genotype 236	5.00±0.00	**MR-MS**
Genotype 237	6.66±0.58	**MS**
Genotype 238	3.33±0.58	**MR**
Genotype 239	5.66±0.58	**MR-MS**
Genotype 24	5.33±0.58	**MR-MS**
Genotype 240	5.33±0.58	**MR-MS**
Genotype 241	5.66±0.58	**MR-MS**
Genotype 242	8.00±0.00	**MS-S**
Genotype 243	3.33±0.58	**MR**
Genotype 244	5.66±0.58	**MR-MS**
Genotype 245	7.00±0.00	**MS**
Genotype 246	2.33±0.58	**R-MR**
Genotype 247	7.66±0.58	**MS-S**
Genotype 248	3.00±1.15	**MR**
Genotype 249	5.00±0.00	**MR-MS**
Genotype 25	6.33±0.58	**MR-MS**
Genotype 250	4.66±0.58	**MR-MS**
Genotype 251	3.33±0.58	**MR**
Genotype 252	7.00±0.00	**MS**
Genotype 253	7.33±0.58	**MS**
Genotype 254	3.66±0.58	**MR-MS**
Genotype 255	7.00±0.00	**MS**
Genotype 257	7.00±0.00	**MS**
Genotype 258	6.66±0.58	**MS**

"Table A.1 (Cont'd)"

Genotype 259	4.66±0.58	**MR-MS**
Genotype 26	5.66±0.58	**MR-MS**
Genotype 260	7.00±0.00	**MS**
Genotype 261	6.00±0.00	**MR-MS**
Genotype 262	7.33±0.58	**MS**
Genotype 265	3.00±0.00	**MR**
Genotype 266	5.66±0.58	**MR-MS**
Genotype 267	4.00±0.00	**MR-MS**
Genotype 269	7.33±0.58	**MS**
Genotype 27	4.33±0.58	**MR-MS**
Genotype 270	4.00±0.00	**MR-MS**
Genotype 271	6.66±0.58	**MS**
Genotype 272	5.66±0.58	**MR-MS**
Genotype 273	5.33±0.58	**MR-MS**
Genotype 274	7.33±1.15	**MS**
Genotype 275	3.33±0.58	**MR**
Genotype 276	5.66±0.58	**MR-MS**
Genotype 277	5.33±0.58	**MR-MS**
Genotype 278	5.00±0.00	**MR-MS**
Genotype 28	6.66±0.58	**MS**
Genotype 280	7.66±0.58	**MS-S**
Genotype 282	5.00±0.00	**MR-MS**
Genotype 283	3.33±0.58	**MR**
Genotype 284	5.33±0.58	**MR-MS**
Genotype 29	5.66±0.58	**MR-MS**
Genotype 3	6.66±0.58	**MS**
Genotype 30	6.66±0.58	**MS**
Genotype 31	7.00±0.00	**MS**
Genotype 32	6.66±0.58	**MS**
Genotype 33	5.66±0.58	**MR-MS**
Genotype 34	6.33±0.58	**MR-MS**
Genotype 35	5.33±0.58	**MR-MS**
Genotype 36	7.66±0.58	**MS-S**
Genotype 37	5.33±0.58	**MR-MS**
Genotype 38	7.00±0.00	**MS**
Genotype 39	6.00±0.00	**MR-MS**
Genotype 4	6.00±0.00	**MR-MS**

"Table A.1 (Cont'd)"

Genotype 40	6.33±0.58	**MR-MS**
Genotype 41	3.66±0.58	**MR-MS**
Genotype 42	7.00±0.00	**MS**
Genotype 43	5.66±0.58	**MR-MS**
Genotype 44	7.33±0.58	**MS**
Genotype 45	6.66±0.71	**MS**
Genotype 46	6.00±0.00	**MR-MS**
Genotype 47	6.66±0.58	**MS**
Genotype 48	5.66±0.58	**MR-MS**
Genotype 49	6.66±0.58	**MS**
Genotype 5	6.66±0.58	**MS**
Genotype 50	5.00±0.00	**MR-MS**
Genotype 51	6.33±0.58	**MR-MS**
Genotype 52	5.66±0.58	**MR-MS**
Genotype 53	6.00±0.00	**MR-MS**
Genotype 54	7.00±0.00	**MS**
Genotype 55	6.66±0.58	**MS**
Genotype 56	5.00±0.00	**MR-MS**
Genotype 57	5.00±0.00	**MR-MS**
Genotype 58	7.66±0.58	**MS-S**
Genotype 59	6.33±0.58	**MR-MS**
Genotype 6	6.66±0.58	**MS**
Genotype 60	5.66±0.58	**MR-MS**
Genotype 61	6.00±0.00	**MR-MS**
Genotype 62	5.33±0.58	**MR-MS**
Genotype 63	3.00±0.00	**MR**
Genotype 64	7.00±0.00	**MS**
Genotype 65	6.00±0.00	**MR-MS**
Genotype 66	7.00±0.00	**MS**
Genotype 67	7.00±0.00	**MS**
Genotype 68	5.33±1.15	**MR-MS**
Genotype 69	6.33±0.58	**MR-MS**
Genotype 7	5.33±0.58	**MR-MS**
Genotype 70	6.00±0.00	**MR-MS**
Genotype 71	5.33±0.58	**MR-MS**
Genotype 72	7.66±0.58	**MS-S**
Genotype 73	6.66±0.58	**MS**

"Table A.1 (Cont'd)"

Genotype 74	7.00±0.00	**MS**
Genotype 75	5.33±0.58	**MR-MS**
Genotype 76	5.66±0.71	**MR-MS**
Genotype 77	5.33±0.58	**MR-MS**
Genotype 78	5.66±0.58	**MR-MS**
Genotype 79	6.33±0.58	**MR-MS**
Genotype 8	7.00±0.00	**MS**
Genotype 80	4.66±0.58	**MR-MS**
Genotype 81	6.33±0.58	**MR-MS**
Genotype 82	4.33±0.58	**MR-MS**
Genotype 83	6.66±0.58	**MS**
Genotype 84	6.66±0.58	**MS**
Genotype 85	3.66±0.58	**MR-MS**
Genotype 86	5.33±0.58	**MR-MS**
Genotype 87	6.66±0.58	**MS**
Genotype 88	6.33±0.58	**MR-MS**
Genotype 89	5.66±0.58	**MR-MS**
Genotype 9	6.00±0.00	**MR-MS**
Genotype 90	5.66±0.58	**MR-MS**
Genotype 91	5.66±0.58	**MR-MS**
Genotype 92	5.00±0.00	**MR-MS**
Genotype 93	5.33±0.58	**MR-MS**
Genotype 94	5.33±0.58	**MR-MS**
Genotype 95	7.66±0.58	**MS-S**
Genotype 96	6.33±0.58	**MR-MS**
Genotype 97	5.00±0.00	**MR-MS**
Genotype 98	7.00±0.00	**MS**
Genotype 99	7.00±0.00	**MS**
Avcı 2002	4.66±0.58	**MR-MS**
Bülbül 89	7.66±0.58	**MS-S**

B. Quality/quantity analyses results of DNA samples

Table B.1 NanoDrop analyses results of DNA samples of DH and parental lines.

Genotype	DNA concentration (ng/uL)	A260/A280 ratio	A260/A230 ratio
Genotype 1	385.9	1.96	2.5
Genotype 10	458.3	1.98	2.52
Genotype 100	226.3	1.98	2.15
Genotype 101	317.7	1.97	2.49
Genotype 102	392.1	1.97	2.32
Genotype 103	318.1	1.98	2.24
Genotype 104	402	1.95	2.37
Genotype 105	397.9	1.97	2.55
Genotype 106	346.4	1.97	2.56
Genotype 107	480	1.97	2.51
Genotype 108	159	1.9	2.17
Genotype 109	168.4	1.91	2.38
Genotype 11	239.6	1.97	2.34
Genotype 110	768.8	1.99	2.45
Genotype 111	169.3	1.92	2.32
Genotype 112	241.4	1.95	2.39
Genotype 113	347.8	1.93	2.42
Genotype 114	339.9	1.98	2.49
Genotype 115	358.3	1.96	2.38
Genotype 116	397.3	1.95	2.42
Genotype 117	301.8	1.96	2.53
Genotype 118	496.3	2.0	2.56
Genotype 119	480.4	1.97	2.45
Genotype 12	430.8	1.98	2.42
Genotype 120	392.7	1.95	2.4
Genotype 121	243.8	1.94	2.35
Genotype 122	468.6	1.96	2.42
Genotype 123	216.6	1.92	2.22
Genotype 124	218.0	1.93	2.28
Genotype 125	178.5	1.94	2.25
Genotype 126	489.3	2.02	2.43
Genotype 127	280.2	1.94	2.41
Genotype 128	201.1	1.93	2.39

"Table B.1 (Cont'd)"

Genotype 129	138.2	1.92	2.45
Genotype 13	154.8	1.96	2.34
Genotype 130	245.4	1.94	2.45
Genotype 131	463.6	1.97	2.44
Genotype 132	337.3	1.92	2.42
Genotype 133	406.8	1.95	2.43
Genotype 134	497.8	1.99	2.47
Genotype 135	218.2	1.94	2.52
Genotype 136	263.4	1.95	2.50
Genotype 137	235.6	1.95	2.51
Genotype 138	119.2	1.92	2.39
Genotype 139	126.2	1.96	2.19
Genotype 14	299.9	1.98	2.35
Genotype 140	737.4	1.95	2.60
Genotype 141	606.3	1.99	2.48
Genotype 142	125.5	1.90	2.09
Genotype 143	273.3	2.01	2.54
Genotype 144	460.3	1.95	2.45
Genotype 145	394.4	1.98	2.28
Genotype 146	306.7	1.98	2.51
Genotype 147	499.7	1.97	2.48
Genotype 148	162.8	1.98	2.48
Genotype 149	400.2	1.98	2.47
Genotype 15	376.0	1.96	2.06
Genotype 150	301.2	1.93	2.32
Genotype 151	398.2	1.97	2.52
Genotype 152	429.4	1.95	2.28
Genotype 153	380.6	1.95	2.40
Genotype 154	329.9	1.94	2.47
Genotype 155	271.3	1.94	2.43
Genotype 156	387.7	1.95	2.48
Genotype 157	339.6	1.98	2.54
Genotype 158	320.8	1.92	2.10
Genotype 159	366.9	1.97	2.52
Genotype 16	598.3	1.97	2.39
Genotype 160	347.0	1.96	2.47
Genotype 161	286.4	1.93	2.40

"Table B.1 (Cont'd)"

Genotype 162	362.9	1.96	2.48
Genotype 163	407.6	1.96	2.41
Genotype 164	325.9	1.94	2.47
Genotype 165	369.1	1.93	2.40
Genotype 166	280.9	1.95	2.32
Genotype 167	307.5	1.96	2.46
Genotype 168	336.1	1.98	2.53
Genotype 169	417.2	1.96	2.46
Genotype 17	386.0	1.95	2.36
Genotype 170	262.9	1.95	2.42
Genotype 171	203.0	1.96	2.38
Genotype 172	200.5	1.98	2.42
Genotype 173	444.8	1.95	2.36
Genotype 174	252.6	1.98	2.53
Genotype 175	320.2	1.97	2.53
Genotype 176	215.9	1.96	2.44
Genotype 177	418.4	1.98	2.53
Genotype 178	234.7	1.97	2.47
Genotype 179	588.1	1.92	2.28
Genotype 18	407.2	1.98	2.47
Genotype 180	159.0	1.96	2.43
Genotype 181	348.1	1.98	2.37
Genotype 182	314.0	1.98	2.51
Genotype 183	459.0	1.95	2.37
Genotype 184	554.3	1.96	2.46
Genotype 185	598.4	1.99	2.38
Genotype 187	519.6	1.98	2.47
Genotype 188	449.3	1.99	2.53
Genotype 189	235.6	1.95	2.29
Genotype 19	203.4	1.97	2.45
Genotype 190	296.3	1.98	2.34
Genotype 191	261.5	1.95	2.28
Genotype 192	293.8	1.96	2.32
Genotype 193	352.1	1.97	2.51
Genotype 194	621.8	1.95	2.41
Genotype 195	277.4	1.90	2.23
Genotype 196	310.2	1.95	2.51

"Table B.1 (Cont'd)"

Genotype 197	278.6	1.97	2.52
Genotype 198	384.7	1.98	2.57
Genotype 199	282.1	1.93	2.15
Genotype 2	370.9	1.96	2.44
Genotype 20	375.6	1.97	2.45
Genotype 200	436.8	1.93	2.40
Genotype 201	555.5	1.97	2.46
Genotype 202	253.8	1.95	2.39
Genotype 203	267.6	1.98	2.49
Genotype 204	307.2	1.96	2.45
Genotype 205	317.9	1.99	2.52
Genotype 206	414.5	1.95	2.47
Genotype 207	348.8	1.94	2.35
Genotype 208	309.3	1.94	2.35
Genotype 209	318.2	1.94	2.34
Genotype 21	354.4	1.96	2.46
Genotype 210	438.9	1.99	2.35
Genotype 211	356.0	1.95	2.36
Genotype 212	435.4	1.96	2.42
Genotype 213	292.7	1.96	2.40
Genotype 214	250.9	1.96	2.37
Genotype 215	263.5	1.94	2.25
Genotype 216	620.5	1.96	2.48
Genotype 217	341.1	1.96	2.47
Genotype 218	277.0	1.96	2.17
Genotype 219	346.1	1.95	2.41
Genotype 22	461.4	1.96	2.49
Genotype 220	301.4	1.96	2.37
Genotype 221	502.4	1.97	2.45
Genotype 222	421.9	1.96	2.51
Genotype 223	480.4	1.97	2.49
Genotype 224	524.3	1.98	2.42
Genotype 225	418.2	1.94	2.41
Genotype 226	221.5	1.98	2.40
Genotype 227	295.0	1.94	2.30
Genotype 228	396.9	1.95	2.39
Genotype 229	624.2	1.98	2.52

"Table B.1 (Cont'd)"

Genotype 23	365.6	1.98	2.41
Genotype 230	498.9	1.96	2.47
Genotype 231	294.8	1.93	2.40
Genotype 232	420.8	1.95	2.43
Genotype 233	457.2	1.96	2.47
Genotype 234	426.2	1.97	2.34
Genotype 235	329.7	1.95	2.42
Genotype 236	332.6	1.94	2.44
Genotype 237	352.8	1.91	2.26
Genotype 238	344.6	1.96	2.34
Genotype 239	349.9	1.98	2.46
Genotype 24	273.0	1.98	2.31
Genotype 240	476.6	1.95	2.40
Genotype 241	222.6	1.99	2.35
Genotype 242	284.6	1.94	2.36
Genotype 243	761.5	1.94	2.39
Genotype 244	391.7	1.98	2.29
Genotype 245	298.8	1.93	2.34
Genotype 246	543.2	1.97	2.49
Genotype 247	227.0	1.97	2.30
Genotype 248	174.9	1.87	2.26
Genotype 249	227.5	1.93	2.27
Genotype 25	326.0	1.95	2.42
Genotype 250	297.6	1.90	2.11
Genotype 251	502.6	1.96	2.45
Genotype 252	223.7	1.97	2.15
Genotype 253	351.1	1.96	2.40
Genotype 254	331.1	1.95	2.40
Genotype 255	517.3	1.97	2.54
Genotype 257	255.4	1.96	2.38
Genotype 258	306.7	1.95	2.35
Genotype 259	617.9	1.92	2.24
Genotype 26	372.4	1.94	2.37
Genotype 260	530.9	1.96	2.43
Genotype 261	636.3	1.95	2.56
Genotype 262	433.3	1.94	2.36
Genotype 265	325.2	1.96	2.45

"Table B.1 (Cont'd)"

Genotype 266	386.4	1.97	2.37
Genotype 267	273.0	1.95	2.35
Genotype 269	426.3	1.88	2.38
Genotype 27	118.2	1.95	2.43
Genotype 270	450.2	1.96	2.46
Genotype 271	285.0	1.94	2.32
Genotype 272	698.1	1.94	2.52
Genotype 273	523.1	1.94	2.28
Genotype 274	375.9	1.96	2.55
Genotype 275	212.0	1.95	2.21
Genotype 276	344.8	1.93	2.36
Genotype 277	370.1	1.97	2.34
Genotype 278	482.0	1.97	2.42
Genotype 28	278.5	1.99	2.34
Genotype 280	575.4	1.99	2.40
Genotype 282	471.9	1.98	2.46
Genotype 283	623.4	1.94	2.48
Genotype 284	307.5	1.95	2.41
Genotype 29	346.1	1.98	2.37
Genotype 3	229.6	1.95	2.31
Genotype 30	489.7	1.98	2.49
Genotype 31	116.5	1.98	2.39
Genotype 32	174.4	1.95	2.21
Genotype 33	423.0	1.99	2.49
Genotype 34	168.7	1.96	2.34
Genotype 35	265.6	1.96	2.52
Genotype 36	292.2	1.97	2.41
Genotype 37	307.5	1.96	2.51
Genotype 38	251.4	1.96	2.41
Genotype 39	420.6	1.97	2.45
Genotype 4	252.4	1.96	2.54
Genotype 40	357.6	1.96	2.45
Genotype 41	264.8	1.97	2.42
Genotype 42	303.2	1.97	2.34
Genotype 43	315.8	2.00	2.51
Genotype 44	211.7	1.97	2.43
Genotype 45	219.8	1.98	2.29

"Table B.1 (Cont'd)"

Genotype 46	431.8	1.96	2.15
Genotype 47	310.2	1.98	2.25
Genotype 48	366.2	1.97	2.34
Genotype 49	262.1	1.96	2.23
Genotype 5	248.6	1.99	2.33
Genotype 50	173.2	1.96	1.99
Genotype 51	354.7	1.98	2.20
Genotype 52	190.7	1.95	2.05
Genotype 53	259.0	1.99	2.50
Genotype 54	165.5	1.95	2.02
Genotype 55	263.6	1.99	2.47
Genotype 56	223.9	1.98	2.14
Genotype 57	384.4	1.97	2.46
Genotype 58	312.8	1.96	2.46
Genotype 59	213.7	1.94	2.35
Genotype 6	186.9	1.99	2.51
Genotype 60	553.0	1.98	2.46
Genotype 61	390.4	1.89	1.97
Genotype 62	244.0	1.94	2.31
Genotype 63	209.9	1.95	2.30
Genotype 64	399.8	1.97	2.45
Genotype 65	540.4	1.96	2.20
Genotype 66	268.8	1.93	2.29
Genotype 67	373.6	1.91	2.24
Genotype 68	424.3	1.94	2.06
Genotype 69	265.3	1.96	2.40
Genotype 7	353.0	2.00	2.29
Genotype 70	372.7	1.96	2.42
Genotype 71	308.5	1.98	2.25
Genotype 72	398.0	2.00	2.42
Genotype 73	364.7	1.90	2.06
Genotype 74	416.4	1.98	2.46
Genotype 75	291.3	1.94	2.27
Genotype 76	340.9	1.97	2.30
Genotype 77	257.0	1.94	2.25
Genotype 78	426.9	1.96	2.46
Genotype 79	249.6	1.93	2.38

"Table B.1 (Cont'd)"

Genotype 8	475.2	1.98	2.53
Genotype 80	408.9	1.94	2.40
Genotype 81	181.3	1.93	2.33
Genotype 82	273.0	1.94	2.33
Genotype 83	301.7	1.88	1.89
Genotype 84	336.8	1.98	2.28
Genotype 85	268.9	1.95	2.04
Genotype 86	309.2	1.95	2.54
Genotype 87	272.7	1.94	2.39
Genotype 88	259.9	1.96	2.12
Genotype 89	297.4	1.95	2.51
Genotype 9	281.7	1.99	2.50
Genotype 90	392.1	1.95	2.18
Genotype 91	203.7	1.92	2.21
Genotype 92	549.7	1.97	2.42
Genotype 93	401.9	1.95	2.09
Genotype 94	306.0	1.88	1.86
Genotype 95	281.5	1.97	2.26
Genotype 96	400.0	1.94	2.09
Genotype 97	215.3	1.96	2.07
Genotype 98	181.3	1.90	2.16
Genotype 99	209.1	1.93	2.29
Avcı 2002	619.7	1.96	2.45
Avcı 2002	596.2	1.96	2.52
Bülbül 89	318.7	1.94	2.34
Bülbül 89	426.2	1.95	2.43

C. Linkage map analysis

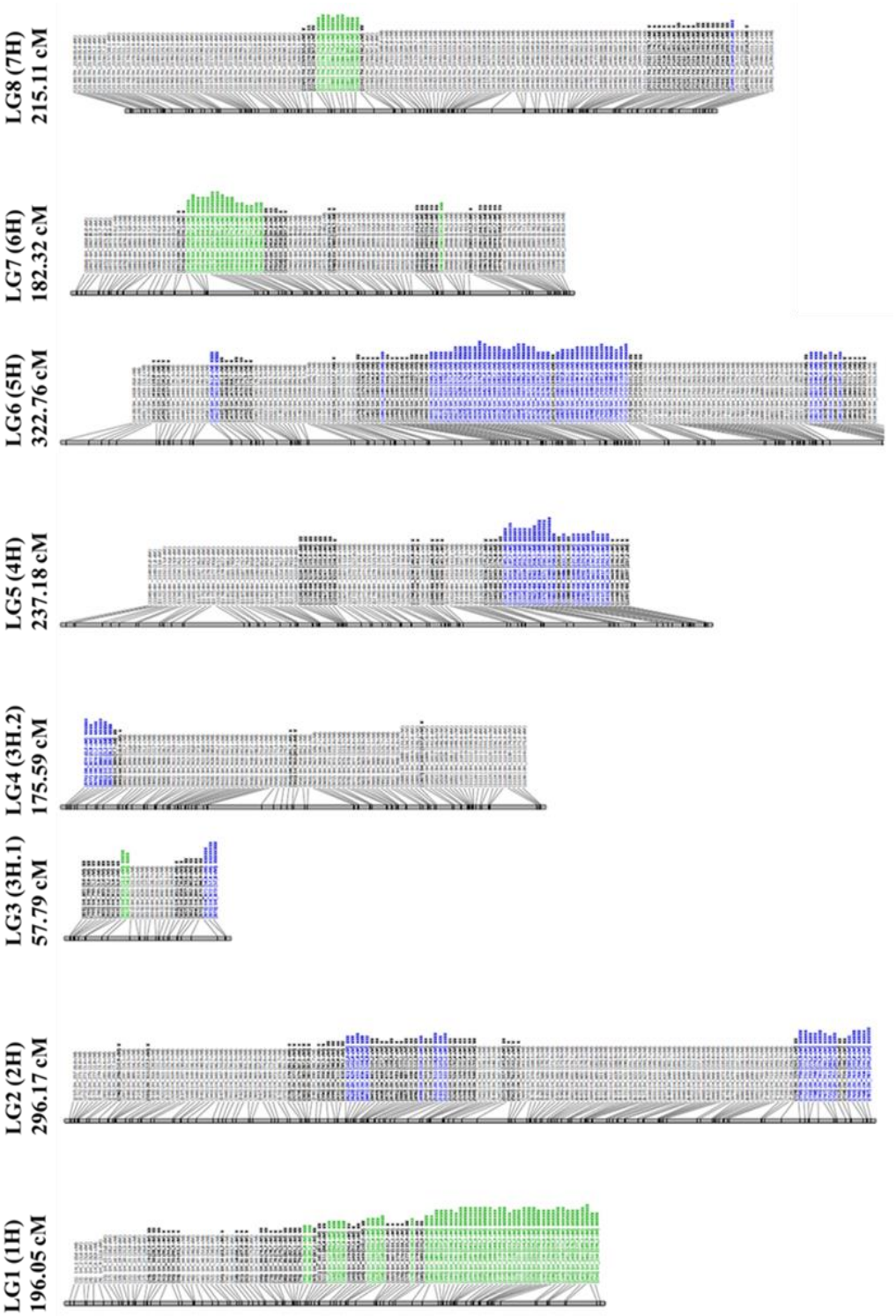

Figure C.1. LGs correspond to the seven chromosomes of barley based on the A × B DH population linkage map.

CURRICULUM VITAE

Surname, Name: Haydaroğlu, Ülkü Selcen

EDUCATION

Degree	Institution	Year of Graduation
MSc	University of East Anglia & John Innes Centre, Plant Genetics and Crop Improvement, Norwich, the UK	2016
MSc	Hacettepe University, Bioengineering, Ankara, Turkey	2015
BS	Ege University, Bioengineering, İzmir, Turkey	2012
High School	Adnan Menderes Anatolian High School, Aydın, Turkey	2008

WORK EXPERIENCE

Year	Place	Enrollment
2016-cont'd	General Directorate of Agricultural Research and Policies, Field Crops Central Research Institute, Ankara, Turkey	R&D Engineer
2015-2016	General Directorate of Agricultural Research and Policies, Norwich, the UK	Ministry of National Education (YLSY) Scholar

FOREIGN LANGUAGES

Advanced English

ARTICLES PUBLISHED IN THE INTERNATIONAL JOURNALS

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