Transposon-assisted functional genomics of malting quality QTLs in barley

Surinder Singh

Department of Plant Science
McGill University
Montreal, Canada

January 2015

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Table of Contents

Abstract

Barley is a significant industrial crop with growing role in feed, food, and bio-products especially as malt for brewing and distilling industry. Consistency in quality of malt is one of the major components in beer production. Malting quality in barley is a multifarious phenotype that combines a large number of interrelated components, each of which has a complex inheritance thus hinder genetic improvement in this important trait. Genetic mapping studies identified more than 250 QTL's (Qualitative trait loci) associated with 19 malting traits. However, limited studies have been conducted to identify genes associated with these QTLs. Map-based cloning and transposons tagging are the main approaches successfully used for gene identification and characterization. Map based cloning is tedious in barley, due to repetitive (>80%) genes and large genome size. Recent successful introduction of *Ac/Ds* (Activator/Dissociation) transposons in barley offers an alternative and promising approach for gene identification. One major QTL complex, QTL2 mapped on the short arm of chromosome 4H, shows large effects on malting quality parameters thus warranted its detailed characterization. Plants with single *Ds* insertions (TNPs), mapping near genes of interest or QTLs, become important vehicles for gene cloning through re-activation of *Ds*. From a publically available barley *Ds* insertion lines, two lines, TNP-29, and TNP-79, map to chromosome 4H, in the vicinity of QTLs for malting quality traits. Reactivation of *Ds* transposon using conventional and tissue culture approaches from these TNP lines lead to the identification of genes harboring in this 4H-QTL regions. Tissue-culture activation approach enabled a threefold faster development of transposon mutants for the genomic dissection of malting QTLs. Identification of unique flanking sequences, using high-efficiency thermal asymmetric interlaced PCR and inverse PCR confirmed *Ds* insertion in genes potentially associated with malting quality such as *β-GAL1* and *β-amylase*-like genes. Alternatively, we also employed a comparative barley-rice synteny approach that detected 24 candidate genes; one of them (*HvTLP8*) which mapped in the QTL2 region shows differential gene expression using Real Time qPCR, among malting and non-malting barley varieties. Biochemical analyses like protein-carbohydrate binding assay, glycosylation and ELISA further confirm the association of *HvTLP8* with QTL2 and malting quality. Integrated effort of

saturation mutagenesis with *Ds* transposons and synteny- based approach will lead to a better understanding of malting quality traits and candidate genes that display quantitative variation

Résumé

L'orge est considéré comme une culture industrielle importante avec le rôle croissant dans l'alimentation, la nourriture et les bio-produits en particulier sous forme de malt pour l'industrie de la distillation et de brassage. Cohérence dans la qualité de malt est l'une des principales composantes de la production de bière. Qualité brassicole de l'orge est un phénotype multiforme, qui combine un grand nombre de composantes interdépendantes liées, dont chacune a un héritage complexe d'empêcher ainsi l'amélioration génétique dans ce trait important. Des études de cartographie génétique identifiée (trait qualitatif loci) plus de 250 de QTL associés à 19 traits de maltage. Des études limitées ont été menées pour identifier les gènes associés à ces QTL. Carte clonage basé sur des transposons et le marquage sont les principales approches utilisées avec succès pour l'identification des gènes et la caractérisation. Carte clonage basé est fastidieux en orge, en raison de (> 80%) des gènes répétitifs et grande taille du génome. Récente introduction réussie de Ac / Ds (Activator / dissociation) transposons dans l'orge offre une alternative et approche prometteuse pour l'identification des gènes. Un complexe majeur d'QTL, QTL2 mappé sur le bras court du chromosome 4H, montre des effets importants sur les paramètres de qualité de maltage ainsi justifié sa caractérisation détaillée. Plantes avec des insertions simples DS (de TNPS), la cartographie des gènes d'intérêt à proximité ou QTL, deviennent des vecteurs importants pour le clonage de gènes à travers la réactivation de *Ds*. A partir d'une ligne publiquement disponibles orge *Ds* d'insertion, deux lignes, TNP-29, et le TNP-79, carte de chromosome 4H, dans le voisinage de QTL pour des caractères de qualité brassicole. Réactivations de Ds transposon de cette ligne TNP mener à l'identification de gènes dans cette région abritant QTL2. Identification des séquences flanquantes uniques, en utilisant la PCR à haute efficacité thermique PCR asymétrique et inverse entrelacé confirmé insertion *Ds* dans les gènes potentiellement associée à la qualité brassicole comme β-GAL1, gène β-amylase comme. Sinon, nous avons également utilisé une approche de synténie orge riz comparative qui a détecté 24 gènes candidats; l'un d'eux (HvTLP8) montre l'expression différentielle des gènes en utilisant qPCR, entre brasserie et non maltage variétés d'orge. Analyses biochimiques comme Reliure

test ELISA et confirme encore et corréler ces résultats avec la quantité de bêta-glucane. Effort intégré de la mutagenèse à saturation avec transposons *Ds* et approche fondée sur les synteny- conduira à une meilleure compréhension des caractéristiques de qualité brassicole et des gènes candidats qui affichent la variation quantitative

Acknowledgements

I would like to express my special appreciation and gratitude to my supervisor, Dr. Jaswinder Singh, whose understanding, expertise, and patience made me complete my graduate studies. Prof. Singh not only initiated me into learning the exciting subject of genomics, but also molded me into an independent researcher. He provided me moral support many times and always encouraged me to think out of the box. Above all, he inspired me with his high scientific standards and problem solving skills.

I would like to further extend my gratitude to my advisory committee members Dr. Philippe Seguin and Dr. Jean-Benoit Charron for their constant support and motivation throughout my research project. Special thanks to Dr. Phil Bregitzer and Dr. Peggy G. Lemaux for providing basic material for my study. Further, all the Singh lab members from past and present; Dr. Manjit Singh, Han Qi-Tan, Prabhjot Nandha, Marie Josée Cardinal, Neil Dylan Lamb-palmer, Dr. Ravneet Kaur, Simerjeet Kaur, Daishu Yi, Bruce Tsai, Sajjad Ahmad, Harvinder Syan, Haritika Majithia and various undergraduate students and visiting scientists, made me feel like having a second family, they owe my sincere acknowledgements for their help and support. I am thankful to my friends and colleagues at plant science department, Alberto Prado, Jamil Samsatly, Mamta Rani, Shivanand Hukkeri and Yevgeniy Zolotarov. Thanks to all of you! This thesis would not have happened without your help. I am also thankful to Prof. Suha Jabaji for allowing me to use their Nanodrop instrument, and Rony Chamoun, for helping me in thesis and for his suggestions during my Ph.D. studies. I thank Prof. Raj Duggavathi and his students especially Dayanand Siddapaa and Yasmin Schuermann for teaching me western blotting.

I wish to thank plant science staff, Lynn Bachand, Carolyn Bowes, Ian Ritchie and Guy Rimmer for their sincere help in administrative and greenhouse assistance. I thank François Gagné-Bourque for his help in translating thesis abstract to French. I am also grateful to all the graduate students of plant science, for nominating me for plant science postgraduate award 2012. Special thanks to Department chair Dr. Philippe Seguin, current graduate program director, Dr. Martina Stromvik and past graduate

program director, Dr. Pierre Dutilleul for their constant support for my graduate study including their support while upgrading from M.Sc to Ph.D. I would also extend my gratitude to the project funding agencies Barley Malting and Brewing Research Institute (BMBRI), The Natural Sciences and Engineering Research Council of Canada (NSERC) and Réseau Innovagrains. Further appreciation for providing various travel awards to attend international conferences by Plant science department, MCGSS, and Centre Séve.

Most importantly this thesis would not have been possible without the love, courage and understanding of my parents and sisters. My grandfather although not physically present always wanted me to see as a doctor and always encouraged me to pursue a doctorate degree. Thanks to you!

Finally, I would also like to thank all of many friends and everyone else who supported me in writing, and incented me to strive towards my goal. A final thanks to God, for making my way through all the difficulties. I will keep on trusting you throughout my life. Thank you, Lord.

List of Tables

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Table 5.1: Table summarizing candidate genes obtained using synteny comparison of QTL2 barley region with rice 3L. It also includes information about the tissue where the EST is expressed, their homology with BLASTx and map location. Map position was confirmed with IPK barley database (http://webblast.ipk-gatersleben.de/barley/).

Table 5.2: Identification of putative *HvTLP* genes from available genome sequence database of *Hordeum vulgare* var. morex (malt) and *Hordeum vulgare* var. bowman (feed). TLP signature for presence (Y), absence (N) or altered (A) sequences is mentioned.

List of Figures

Chapter 2

Chapter 3

marker. H_2O and GP are negative controls; original TNP-29 and TNP-79 lines are used as positive controls. Arrows indicate the expected band size of each PCR amplification.

Figure 3.5: Diagrammatic illustration of flanking sequences showing the positions of exon, intron, the *Ds* insertion site, genomic regions, the putative start and stop codon and domain positions.

Chapter 4

Figure 4.1: Diagrammatic representation of additional flanking sequences generated, showing the positions of exon, intron, the *Ds* insertion site, genomic regions, the putative start and stop codon and domain positions.

Figure 4.2: a) *Ds* Transposon insertion line MTNP 301, showing polymorphism as detected using a CAPs assay. A SNP detected in one of mapping parent (OWB-R) has a restriction enzyme (sau1) site, which restrict all the mapping population individuals with 'OWB-R type' and not the 'OWB-D type'. b) MTNP 310 showing a polymorphism detected in Steptoe X Morex population due to a SNP. Fifteen samples are shown here in each population as an example

Figure 4.3: Barley linkage map showing MTNP lines located on Chromosome 1H to 7H as inferred from barley sequence database. Each MTNP line mapped on individual chromosome is mentioned below. Red arrows indicate the location of MTNP lines.

Figure 4.4: Confirmation of mapped position of MTNP lines (MTNP 301 and MTNP 310) with genetic linkage map constructed using Joinmap4. Red arrows indicate the position of MTNP lines.

Figure 4.5: Frequency of MTNP lines location across different barley chromosomes**.**

Chapter 5

Figure 5.1: Figure depicts the conserved syntenic region between barley chromosome 4HS and rice chromosome 3L. A segment (0-15cM) on short arm of barley chromosome 4H represents QTL2 which is syntenic and maintains co-linearity with a region on chromosome 3L of rice. Figure designed using strudel software.

Figure 5.2: Figure a) and b) represents phylogenetic relationship showing the relatedness of the deduced full length amino acid sequence of barley HvTLP8. The Phylogenetic tree was generated using MEGA 6 program. The scale represents the frequency of amino acid substitution between sequences as determined by poisson evolutionary distance method.

Figure 5.3: a) Structure of *HvTLP8* mature mRNA, with a scale 0 to 2500 bp. Various regions with their sequence lengths are indicated. b) Presence of an acidic pocket (red) in the three dimensional structure of HvTLP8 protein. This pocket is created at the motif site from beta-hairpins, and is believed to be involved in binding of β-glucan. c) Motif (CQTGDCGG) with possible glycosylation site.

Figure 5.4: Secondary structure prediction of HvTLP8 in; a) Morex (malting variety) and b) Bowman (feed variety), based on data obtained from barley whole genome sequencing project. Amino acid sequence showing structural modifications are indicated with a key. c) Dark black outlined box indicate the presence of different amino acid (V) in Morex and (G) in Bowman varieties. Protein comparison shows a normal TLP signature in morex (green) and altered (yellow) in bowman barley variety.

Figure 5.5: a) Gel representation of expression profiles of *HvTLP8* and housekeeping gene (β-actin) among malting and feed varieties using semi-quantitative RT-PCR at different stages of germination. b) Bar graph represents qRT-PCR expression profile of *HvTLP8 gene* in duplicates. Ac Metcalfe, Bentley and Morex are the malting varieties which are up-regulated whereas Cowboy, Coalition and Steptoe are feed varieties which are down-regulated during 16h of germination.

Figure 5.6: Amount of β-glucan at different stages of germination (0hrs, 16hrs and 48hrs). All the malting varieties have lower amount of initial β-glucan as compared to feed varieties. As the germination stages progress to 16 and 48hrs, the amount of β-glucan considerably drops in all the malting varieties, whereas the decrease in feed varieties is significantly less. X-axis represent the malting (1-3) and feed (4-6) and control, whereas Y-axis represent the amount of β-glucan in percentage (w/w).

Figure 5.7: Total proteins were extracted from different varieties of barley seeds, and TLP were precipitated using ammonium acetate precipitation and resolved on SDS PAGE. a) Glycosylation of malting and non-malting varieties observed by staining with glycosylation staining kit. Malting varieties show a high degree of florescence, depicting higher β-glucan binding than non-malting. b) SDS PAGE gel showing TLP after binding with β-glucan. c) Immunoblotting of TLPs with anti-TLP antibody on a nitrocellulose membrane. A small size difference between the bound and unbound proteins in case of malting and no differences in non-malting varieties was observed. 1-6 are malting varieties and 7-12 are non-malting. Bands 1, 3, 5, 7, 9, 11 are normal proteins and 2, 4, 6, 8, 10, 12 are β-glucan bound proteins.

Figure 5.8: ELISA based biochemical assay designed to determine the binding ability of HvTLP with β-glucan (pachyman) and effect of different sulfhydry groups on the binding capacity of HvTLP8 and β-glucan. X axis represent Ac Metcalfe, Bentley and Morex as malting varieties and Cowboy, Coalition and Steptoe as feed varieties, with various reducing agents as treatments and Y-axis represent amount of protein. Bound proteins were calculated by subtracting the total TLPs from the unbound left in the supernatant. Group 1 represent normal unbound protein, group 2 is proteins bound to 5% β-glucan, group 3 is 5mM DTT, group 4 is 0.5mN DTT, group 5 is 10mM reduced glutathione and group 6 is 10 mM β-mercaptoethanol.

List of Abbreviations

AA	α-amylase activity
Ac	Activator
AcTPase	Activator transposase
BAC	Bacterial artificial chromosome
BG	β- glucan content
BLAST	Basic Local Alignment Search Tool
CAPS	Cleaved Amplified Polymorphic Sequence
DArT	Diversity Arrays Technology
DNA	Deoxyribonucleic acid
Dor	Dormancy
DP	Diastatic power
Ds	Dissociation
ELISA	Enzyme-linked immunosorbent assay
EMS	Ethyl methanesulfonate
EST	Expressed sequence Tags
FAO	Food and Agriculture Organization of the United Nations
FAOSTAT	Statistics Division of FAO
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein

GO	Gene Ontology
GP	Golden Promise
HE-TAIL	High Efficiency Thermal Asymmetric Interlaced
IBGSC	International Barley Genome Sequencing Consortium
INDEL	Insertion deletion polymorphism
iPCR	inverse PCR
KP	Kernel plumpness
LOD	Logarithm of odds
MBC	Map based cloning
ME	Malt extract percentage
NABGP	North American Barley Genome Project
NCBI	National centre of biotechnology information
ORF	Open reading frames
OWB	Oregon wolfe barley
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PR	Pathogenesis-related
PVDF	Polyvinylidene fluoride
qPCR	Quantitative real-time PCR
QTL	Qualitative trait loci
RAD	Restriction Site Associated DNA

RAPD	Random amplified polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcription - PCR
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeats
TBS-T	Tris-Buffered Saline and Tween 20
TILLING	Targeting Induced Local Lesions in Genomes
TIRs	Terminal inverted repeats
TLPs	Thaumatin like proteins
TNP	Transposon insertion line
UTR	Untranslated region
VIGS	Virus-induced gene silencing
WGS	Whole genome sequencing

Preface and Contribution of Authors

This thesis is written in the form of the manuscript according to the “Guidelines Concerning Thesis Preparation” of McGill University. It contains three chapters (III to V) representing three separate manuscripts, all of which are either published or in the submission process. Each co-author is mentioned, along with his/her corresponding address at the beginning of each chapter. Below is a general description of the contribution of each author. A detailed description of the contribution is provided in the connecting statements at the beginning of each chapter.

My role in all the chapters was to design experimental strategies, conduct all experimental procedures including bioinformatic analysis and the preparation of the first draft of each manuscript. Dr. Jaswinder Singh provided supervision, technical assistance and funds throughout this study. He contributed significantly to the edition and correction of several versions of the manuscripts. Han Qi Tan as a second author helped in performing tissue culture experiments in Singh et al., 2012 (chapter III). Part of the results from chapter IV has been accepted for a combined publication with our collaborator, Dr. Phil Bregitzer (Brown R, Singh J, Singh S et al., 2015).

Contribution to Science

The chapters in this thesis represent a significant contribution to knowledge for genomic, transcriptomic and biochemical understanding of malting quality in barley.

I used a unique transposon based approach as a vehicle to dissect important malting QTLs. This study is first of its kind where *Ac/Ds* transposons element was employed to explore important QTLs. I also present here a tissue culture based approach to efficiently saturate QTL region. Our data indicate a three-fold increase in transposition frequency when using this tissue culture approach compared to conventional hybridization. Using colinearity and transposon based *Ds* reactivation approaches we identified a gene (*HvTLP8*) which is harboured in QTL2 region and significantly influences malting quality.

In addition we successfully identified functional markers for breeding of barley varieties with enhanced malting quality. Following are the major contributions to knowledge generated from this thesis:

1. New *Ds* transposon lines developed using conventional and tissue culture techniques will be novel for the barley community to further the explore barley genome.

2. Understanding of QTL2 can lead to development of new molecular breeding tools for enhancing malting quality of barley. We developed 41 new *Ds* insertion mutant lines, from which 22 flanking sequences were generated. Some of these were made homozygous and add to the repository of TNP lines developed in the barley genome and were published in NCBI database (GenBank KF466261-KF466270). Additions of these new lines will expand the *Ds* insertion library as functional genomics tool to understand the barley genome.

3. The study also describes the in-vitro activation of the *Ds* element using transient expression of *AcTPase*, which increased the transposition frequency by threefold as compared to conventional approaches. This study will further create a new

transposon-based platform for cloning genes in QTLs in other large genome species, and facilitate transgenic breeding to create marker free transgenic.

4. The QTL2 region was saturated with *Ds* insertion lines, creating a resourceful updated genetic linkage map, further exploring malting and other genes in this QTL. This linkage map provides useful information for candidate malting genes that may be used to study expression analysis, and can further be used by the barley community.

5. A comparative genomic approach with rice generated 100+ genes, 24 on chromosome 4H. The majority have shown polymorphism, and can be used as molecular markers for enhanced breeding of malting traits. We also identified a malting gene, *HvTLP8* in QTL2, which consists of a unique carbohydrate binding motif (G-x-[GF]-x-C-x-T-[GA]-D-C-x(1,2)- [GQ]-x(2,3)-C). This motif binds to β-glucan and sequesters it from the malt. In this context, an ELISA based biochemical assay was developed to characterise the binding competency of HvTLP8 with β-glucan.

6. We also identified genes related to germination and dormancy such as ARGONAUTE4_9, which plays an important role in epigenetic control of pre-harvest sprouting resistance in wheat and barley (Singh et al., 2013)

7. Overall, these studies created a new transposon-based platform for cloning other quality QTLs in large genome species.

<u>This thesis study generated the following publications and manuscripts:</u>

Singh S, Tan H-Q, and Singh J (2012) Mutagenesis of barley malting quality QTLs with *Ds* transposons. **Functional & Integrative Genomics** 12:131-141.

Brown R H, Singh J, **Singh S**, Dahleen L S, Lemaux P G, Stein N, Mascher M, Bregitzer P (2015) Behavior of a modified Dissociation element in barley: A novel tool

for genetic studies and for breeding transgenic barley. **Molecular Breeding** 35: 85 (DOI 10.1007/s11032-015-0193-9).

Singh S, Singh J (2015) Barley Thaumatin-Like Protein gene *HvTLP8* resides in QTL2 and its expression regulates the availability of β-glucan during grain germination. **Proceedings of National Academy of Sciences** (To be submitted**).**

Singh S, Nandha P**,** Singh J (2015) Insertion site structure of mapped barley *Ds* loci of QTL2 region **Genome** (In preparation)**.**

Other related publications:

Singh M, **Singh S**, Randhawa H, Singh J (2013) Polymorphic homoeolog of key gene of RdDM pathway, ARGONAUTE4_9 class is associated with Pre-harvest Sprouting in wheat (*Triticum aestivum* L.) **PLoS ONE** 8(10): e77009. doi:10.1371/journal. pone.00 7 7009.

Nandha P, **Singh S**, Singh J (2015) Transposons based genetic diversity assessment in wild and cultivated barley. **Canadian Journal of Plant Science** (to be submitted).

Chapter I

Introduction

Barley is an important cereal which ranks fifth globally in respect to its total production among other cereal crops. In addition to its importance in agriculture, it has been a model crop for genetics due to its diploid nature and boasts excellent resources for forward genetics, with a large number of mutants available. Barley is also a key industrial crop due to its growing role in feed, food, and other bioproducts. Malt generated from barley is the most important commodity due to its use in the brewing and distillation industries. Consistency in the quality of malt is one of the major factors that brewing industries rely upon for beer production. Malting quality in barley is a multigenic trait, that combines a large number of interrelated components, each of which has complex inheritance (Hayes & Jones 2000). These complexities hinder genetic improvement in this important trait. Genetic mapping studies have identified more than 250 QTL's (Qualitative Trait Loci) associated with 19 malting traits (Wei et al., 2009) across the seven barley chromosomes. Limited studies have been conducted to identify genes associated with these QTLs. One major QTL complex, QTL2 on the short arm of chromosome 4H (between markers MWG634 and BCD402B), shows large effects on malting quality and has, therefore, been characterized in detail (Gao et al., 2004). This QTL2 affects such malting quality parameters as malt extract percentage (ME), α-amylase activity (AA), diastatic power (DP), β- glucan content (BG), kernel plumpness (KP) and dormancy (Dor) (Swanston and Ellis 2002; Hayes et al*.,* 1993)

Map-based cloning and transposon tagging are important approaches for gene identification and characterization. The large genome size of barley and a preponderance of non-transcribed sequences (>80% genes) (Caldwell et al., 2004) make map-based cloning rather tedious and inefficient. The recent successful introduction of *Ac/Ds* (Activator/Dissociation) transposons in barley offers an alternative and promising approach for gene identification (Singh et al., 2006). Plants with single

Ds insertions (TNPs) mapping near genes of interest or QTLs are important vehicles for gene cloning through re-activation of *Ds*. From publically available barley *Ds* insertion lines, two lines, TNP-29, and TNP-79, map to chromosome 4H, in the vicinity of QTLs for malting-quality traits. In TNP-29, *Ds* locus maps close to an important malting quality QTL, QTL2. Similarly, *Ds* locus from TNP-79 mapped in the vicinity of another malting QTL on chromosome 4H. As *Ds* transposons prefer to move into linked genic regions (Cowperthwaite et al., 2002; Kolesnik et al., 2004; Singh et al., 2006; Randhawa et al., 2009), reactivation of Ds in TNP-29 and TNP-79 may lead to identification of important genes harbored in the malting quality QTLs. In this study, our aim was to generate a series of new TNP lines in order to saturate the malting quality QTLs with *Ds* transposons. Conventional reactivation of *Ds* transposons and transient expression of an extra-chromosomal transposase (*Ac*TPase) gene was performed through genetic transformation. Bioinformatic analysis of the new *Ds* flanking sequences provided information on the *Ds* insertion site into a gene.

An alternative approach using synteny-based exploration of QTL2 was also employed. We investigated Barley-Rice synteny, specifically in the QTL2 region. Given that the rice genome is fully sequenced, better annotated than barley, and possesses numerous genetic resources, it was chosen as the reference model species for syntenic studies with barley. Candidate genes affecting malting quality, generated from *Ds* insertional mutagenesis and syntenic approaches, have been mapped and examined for their expression profiles. Differential expression across different barley varieties used as malt or feed was carried out using semi-quantitative RT-PCR and real-time qPCR analysis. Subsequently, expression analysis, protein profiles and biochemical assays of an important malting gene, *HvTLP8* located in QTL2 region were carried out.

The overall goal of this project was to dissect the QTL2 region, an important malting quality QTL, with different strategies such as studying expression profiles and protein analysis of the candidate genes. Integrating saturation mutagenesis with *Ds* transposons and with synteny-based approach led to a better understanding of malting quality traits and candidate genes that display quantitative variation in their expression.

1.1 Hypotheses

1. The QTL2 region of chromosome 4H in barley contains genes that affect malting quality.

2. The *Ac/Ds* transposon system is an effective tool for dissecting QTL regions, and *Ds* elements tend to transpose to genetically linked positions.

3. Barley-rice synteny can provide putative gene information for a known QTL.

4. Genes associated with the QTL2 region have altered expression and are differentially expressed across malting and feed barley varieties.

5. Differentially expressed candidate genes will have altered protein profiles.

1.2 Objectives

1. Saturation of the QTL2 region by reactivation of *Ds* transposons from candidate TNP lines (TNP-29 and TNP-79) using conventional and tissue culture approaches.

2. Generation of *Ds* flanking sequences from newly developed TNPs and their basic bioinformatic analysis.

3. Identification of SNP/INDEL polymorphism in the parents of mapping populations and localization of polymorphic *Ds* flanking sequences on barley chromosomes.

4. Exploration of the QTL2 region for identification of putative genes and their orthologs using Barley-Rice synteny.

5. Expression analysis of candidate malting quality genes identified using *Ac/Ds* insertional mutagenesis and synteny approach, in malt and feed barley varieties.

6. Studying protein profiles from differentially expressed malting genes, using immunoblotting and other biochemical assays (i.e. ELISA and glycosylation analysis).

In this thesis, we tested all the hypotheses and fulfilled all the objectives mentioned above. Chapter 3 deals with objectives 1 and 2, chapter 4 with objective 3 and chapter 5 with objectives 4, 5 and 6.

Chapter II

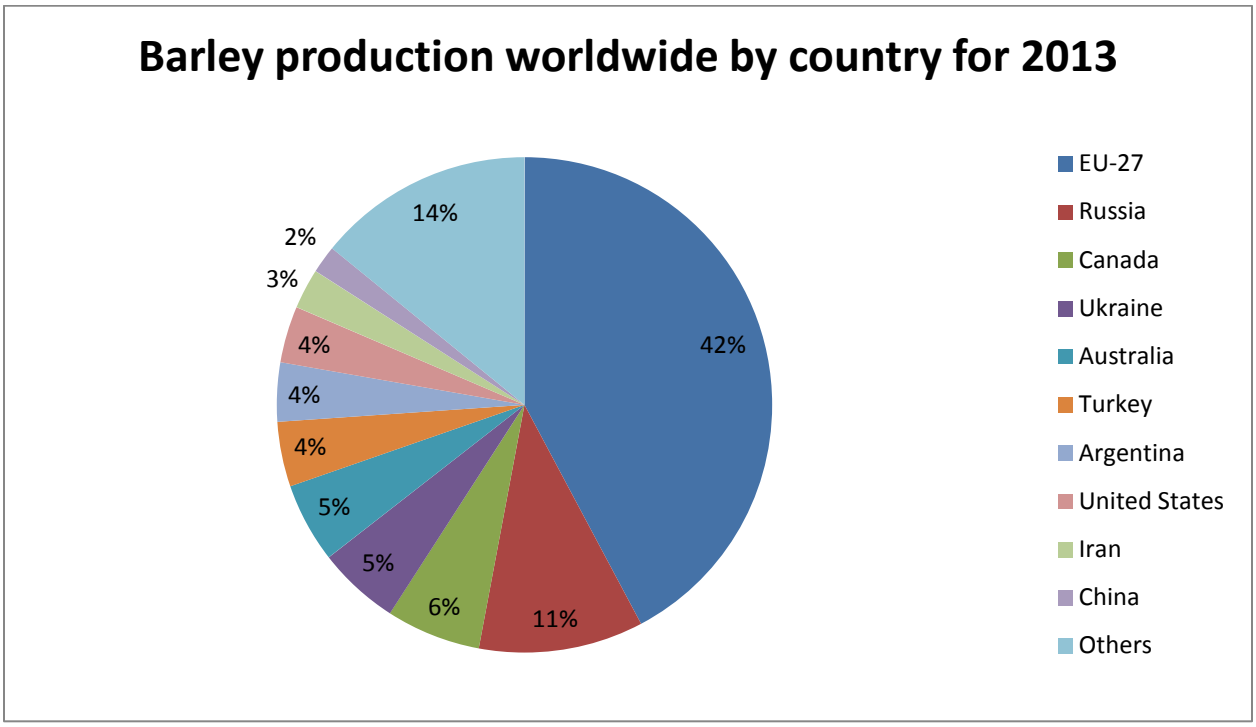
Literature Review

2.1 History and importance of barley

Barley (*Hordeum vulgare* subsp. *vulgare)* is one of the oldest cereal crops grown (Badr et al., 2000; Pourkheirandish and Komatsuda 2007). Archeological remains indicate that barley was domesticated around 8000 B.C (Zohary and Hopf 1993) at various sites in the Fertile Crescent. Barley is cultivated throughout the world, in very diverse environments such as the arctic of Northern Europe, African subtropical regions and the highlands of the Himalayas (Stanca 2003). Barley belongs to the Poaceae family and originated from its wild progenitor *Hordeum vulgare* subsp*. spontaneum.*

Barley ranks fourth in world cereal production, after wheat, rice and maize with a total production of 132.35 million tons (FAOSTAT 2013). Canada is the third largest producer of barley with total production of 8.02 million tons (FAOSTAT 2014) (Figure 2.1). It is mainly used as a feed crop and as raw material (malt) for brewing and distillation. Canada is among the top three producers and exporters of malted barley. Traditionally, barley has also been used as food after dehulling, polishing, and milling (Bhatty, 1992). In Japan and Korea, whole polished barley seed is still used as a substitute for rice. Use of barley as food is limited although it is increasing due to its various health benefits. This increased recognition of its food value should further increase its market demand and lead to increased production of barley as a food crop.

Figure 2.1: World barley production, 2013 (USDA 2014).



2.2 Barley genome

Barley, with a diploid (2n=2x=14) genome size of 5.1 Gb - is a genetic model for the members of Triticeae. An estimated 24,154 genes have been localized in a physical/genetic scaffold, representing a gene density of five genes per Mb (The International Barley Genome Sequencing Consortium- IBGSC 2012).

About 80% of the barley genome consists of highly repetitive DNA that is mostly retrotransposons. Barley genome, although apparently large, is smaller compared to that of other cereal species that share synteny with barley, such as wheat (~5500Mbp – 18000 Mbp) (Gill et al., 2004), oat (11315 Mbp) (Arumuganathan and Earle, 1991) and rye (7917 Mbp) (Bartos et al., 2008). Genetic resources in barley have expanded exponentially in recent years. Just over forty years ago, linkage data were available for only 79 loci in barley (Nilan 1964). Currently, the barley map contains a large number of

genetic markers, including RFLPs, SSR, DArTs, and SNPs (Paux et al., 2009, Munoz-Amatriain et al., 2011). Recently, high-density genetic maps have also been created (Munoz-Amatriain et al., 2014). Further, a consensus SNP map with 2943 loci is available at HarvEST (www.harvest-web.org). Most recently, a sequence-ready physical map of barley was constructed, with genetical anchored two million SNPs and 9,265 contigs with a cumulative size of 4.9 Gb representing 96% of the physical length of the barley genome (Ariyadasa et al., 2014).

2.3 Genetic markers in barley

A genetic marker can be defined as a chromosomal landmark or allele that enables tracing of a specific region of DNA (King and Stansfield 1990). Markers tightly linked to the gene of interest can be indirectly used to select the desired allele (Beecher et al., 2002). Genetic markers can be categorized into three main classes: those based on visually assessable traits (morphological and agronomic traits), biochemical markers which include allelic variants of enzymes (isozymes) and DNA (or molecular) markers which reveal sites of variation in DNA sequence (Jones et al., 1997; Winter & Kahl 1995). Genetic markers can also be grouped based on the mode of gene action (dominant or co-dominant markers) and method of analysis (hybridization-based or PCR-based markers).

Morphological markers include visually characterized phenotypic characters such as flower color, seed shape, growth habits or pigmentation (Jones et al., 1997). Many morphological markers have been characterized in barley e.g. rachilla hair length, leaf blade pubescence, wide outer glumes, six row head, white streaks in young seedlings, etc. Recently, short rachilla hair, a morphological marker controlled by the recessive gene *srh* has been fine-mapped (Javaid et al., 2009). As morphological markers are limited in number and highly influenced by the environment in their expression, other forms of markers have also been developed.

Biochemical markers include allelic variants of enzymes (Isozymes) and proteins, and differences among them can be detected by electrophoresis and staining. In barley, development of biochemical markers led to the identification of associations between

some important genes related to malting (Forster et al., 1991). Finnie and Svensson (2009) identified polymorphic protein polypeptides by 2-D electrophoresis that have been helpful in differentiating malting barley and feed barley varieties.

DNA-based markers can be hybridization-based, like RFLP, PCR-based like RAPD or sequence-based like microsatellites and SNPs. The RFLP technique is based on restriction enzymes that reveal a pattern difference between DNA fragment sizes in individual organisms. RFLP markers have high reproducibility, co-dominant inheritance, and good transferability between laboratories and have been used to construct genetic maps in barley (Graner 1991, Stein et al., 2007). RAPD markers were generated by using random primers, 10–20 nucleotides long, to detect complementary sites across relatively short distances within the genome (Jones et al., 1997). RAPD markers have played an important role in linkage analysis of barley (Giese et al., 1994). They were used for polymorphism detection, genetic identification and genetic diversity among barley cultivars (Fernández et al., 2002). Similar studies were conducted by Baum et al., (2004) to explore genetic diversity in wild barley (*Hordeum vulgare subsp.* spontaneum). PCR-RAPD markers were constructed to enable differentiation of closely related six-rowed malting barley (*Hordeum vulgare* L.) cultivars (Hoffman et al., 1996).

Other sequence-based markers include microsatellites, also referred to as Simple Sequence Repeats (SSRs), which are typically 2–8 bp long nucleotide repeats in the genome. In the last decade, a large number of SSR markers have been developed in barley (for review see Hearnden 2007). Many EST-based SSR markers have also been developed in barley, designed to detect gene sequences expressed during grain development (Emebiri et al., 2009). SSRs are excellent markers for fluorescent techniques, multiplexing, and high throughput analysis. They have been used to assess genetic diversity among wild and cultivated barley genotypes (Nandha et al., 2014). SSR markers have also been used to establish a variety identification system for Canadian malting barley (Perry et al., 2013).

SNPs are single nucleotide changes that occur in coding and non-coding regions of the genome. SNP markers are likely to be predominantly used in genetic studies of barley and wheat in the near future (Lehmensiek et al., 2009).

Several techniques are used for the identification of large numbers of SNPs in any plant species. The techniques include SNP identification based on EST data, array analyses, amplicon re-sequencing, next-generation sequencing and sequenced whole genomes (Ganal et al., 2009).

2.4 Genetic linkage mapping

Molecular markers have been used to construct genetic linkage maps. Frequency of recombination events or crossovers between markers determine the position and distance between markers, thereby giving a graphical representation of the arrangement of markers along chromosomes to create genetic maps (Collard et al., 2005). Recombination is less likely to occur between markers that reside closer together as compared to markers that are further apart. Distances between markers are measured in centimorgans (cM) where 1 cM is approximately equal to 1 percent chance that two markers will be separated by recombination during meiosis. Four major steps are involved in the construction of a genetic linkage map: (1) production of a mapping population; (2) polymorphism assessment; (3) genotyping the population with polymorphic markers and (4) linkage analysis (Lehmensiek et al., 2009).

A large number of markers used to create linkage maps require use of computer programs. It is also possible to manually determine position and distance between markers for a small number of markers. Many programs are available to perform linkage analysis, for example: Mapmaker/EXP (Lander et al., 1987); Join Map (Stam 1993); MapManager QTX (Manly et al., 2001); Multipoint (Mester et al., 2003); CarteBlanche (Keygene, Wageningen, The Netherlands) and RECORD (Van Os et al., 2005a). Early genetic linkage maps were based on RFLP markers (Devos and Gale 1992 ; Nelson et al., 1995); but with the advent of PCR-based techniques, these were superseded by RAPD (Devos and Gale 1992), AFLP (Vos et al., 1995), SSR (Gupta et al., 2002), SNP (Rostoks et al., 2005b) and DArT markers (Jaccoud et al., 2001).

Recently, high-density genetic maps based on two enzyme-genotyping by sequencing (Poland et al., 2012) and Restriction Site Associated DNA (RAD) linkage maps (Chutimanitsakun et al. 2011) were developed in barley. Most barley linkage maps are

curated and are available on the GrainGenes website (http://wheat.pw.usda.gov/GG2/maps.shtml#barley; verified, 2014). With the combined use of chromosome sorting, next-generation sequencing, array hybridization and exploitation of conserved synteny of barley with model grasses, nearly 86% of the estimated 32, 000 barley genes have been assigned to individual chromosome arms (Mayer et al., 2009, 2011, Matsumoto et al., 2011). Most recently, iSelect array integrated 11 genetic maps to develop a consensus map with iSelect SNP map (Munoz-Amatriain et al., 2014). The map and supporting data are available at the Triticeae Toolbox (T3) database (Available: http://triticeaetoolbox.org/barley/; accessed December 2014). Furthermore, a sequence-ready physical map of barley anchored with 2 million SNPs and covering 96% of the physical distance was constructed using whole genome shotgun sequencing. These resources will significantly simplify the development of molecular markers linked to economically important genes for use in marker-assisted barley breeding.

2.5 Functional genomics in barley

A large number of functional genomics tools, such as mutants, bacterial artificial chromosome (BAC) libraries, ESTs, physical maps, sequencing and bioinformatic data, are now available for barley. This includes 5,56,070 ESTs (NCBI-dbEST summary), Affymetrix DNA chips (Close et al., 2004), TILLING population (Sreenivasulu et al., 2008), chemical and genetic mutants (Waugh et al., 2006), and transposon insertion lines (Singh et al., 2006, Zhao et al., 2006 , Brown et al., 2014, Brown et al., 2015, Singh et al., 2012). The rapid increase in EST collections provides new opportunities for SNP-based and other markers. To provide fundamental information about functional and structural aspects of the barley genome, the International Barley Genome Sequencing Consortium (http://barleygenome.org/) has fully sequenced the barley genome (IBGSC 2012).

Two different strategies can be used for functional genomics, ‘forward genetics’ (from phenotype to gene) and ‘reverse genetics’ (from gene to phenotype). Characterizing gene function through reverse genetics using mutant populations has been developed in barley, and related crops (for review see, Waugh et al., 2006). This involves creating

mutants to screen for phenotypes. Functional genomics techniques like Targeted induced local lesion in genome (TILLING) (Caldwell et al., 2004, Waugh et al., 2006), Virus-induced gene silencing (VIGS) (Holzberg et al., 2002), Transient-induced gene silencing (TIGS) (Douchkov et al., 2005) and insertional mutagenesis using transposons (Singh et al., 2006) has been explored in barley for gene identification. Insertional mutagenesis approach shows great potential in barley as loss-of-function mutations by the insertion of transposable elements into a gene of interest (Koprek et al., 2000; Singh et al., 2006; Zhao et al., 2006; Lazarow et al., 2009) and dominant gain-of-function mutations with activation tagging have been successfully accomplished (Ayliffe et al., 2007, 2009). However, chemical mutagenesis approaches, such as TILLING have been less suitable for creating mutations to saturate a particular region or a QTL, because chemical mutagenesis cannot be directed to a specific site on a genome i.e. there is randomness in induced mutations. Another factor is that all genes may not be equally susceptible to EMS mutagenesis.

In plants, particularly in barley, Map based cloning (MBC) and transposon tagging are the methods of choice for gene identification, characterization and cloning. In spite of some disadvantages with map-based cloning, like large genome size, nontranscribing and repeating sequences, it has been used to discover some significant genes, like those conferring disease resistance in barley.

Many genomic resources are available to assist positional cloning in barley, such as detailed genetic maps (e.g. Hearnden et al., 2007; Varshney et al., 2007; Stein et al., 2007; Sato et al., 2009; Close et al., 2009;) a synteny-informed virtual linear gene-order map (‘‘Genome Zipper’’; Mayer et al., 2011) and a sequence-substantiated physical map (IBGSC 2012)

2.6 Map-based cloning (MBC)

The forward genetics approach for gene isolation is based on MBC (for reviews see Peter et al., 2003). MBC is a strategy to clone genes of interest without prior knowledge of the gene product. A suitable high density genetic map and the ability to perform chromosomal walking on large genome sequences (Feuillet et al., 2003) are important

prerequisites for utilizing MBC. Establishment of relationships among major grass species, especially at the micro level, has made it possible to do MBC of genes in barley by utilizing the results of genetic and physical mapping of the smaller genomes. Disease resistance genes have been cloned in barley using map based cloning.

The first barley gene cloned by the MBC was the recessive *mlo* gene which confers broad spectrum resistance to powdery mildew (Büschges et al., 1997). The barley stem rust resistance genes *rpg1* and *rpg4* were mapped in barley on chromosomes 1P and 7M using MBC (Kilian et al., 1997). Recently, *vrs 1* (Komatsuda et al., 2007) and *Ryd3* (Lüpken et al., 2013) have been characterized through MBC. This approach is still valuable as is evident by the relatively recent isolation of genes of agronomic importance from large genome species such as wheat by advances in positional cloning techniques (Yan et al., 2003; Krattinger et al., 2009).

Due to the large and repetitive genome of barley (Peters et al., 2003), methods using chromosome walking and marker development are inherently difficult and inefficient. Another gene cloning approach is based on insertional mutagenesis using transposons. This technique has a huge scope in cloning important genes and in functional studies of large genome cereals (Singh et al., 2006).

2.7 Transposons

Transposons are repetitive sequences that can move from one genomic location to another throughout the host genome. The movement may happen on the same DNA strand, or even on different DNA strands or chromosomes; this movement is called transposition. In order to transpose, transposons require enzymes such as transposase. The transposon itself usually codes for these proteins (Jones 2005). Transposon movements create mutations by inactivating the genes into which they are inserted usually creating recessive loss-of-functions mutations. In some cases, the insertions may also enhance the phenotypic expression of the gene (Ramachandran, Sundaresan 2001). Recent discoveries have also supported the importance of transposons as a major contributor in evolution (Biemont and Vieira 2006).

Barbara McClintock discovered the first endogenous transposable elements in maize in 1940s (Jones 2005). She discovered that the stationary, non-changing heritable units, were able to move by jumping to another region in the same or different chromosome. She identified two dominant genetic loci that she called *Ds* (Dissociator) and *Ac* (Activator) in maize (Jones 2005).

Transposons are divided into two categories, class I and class II elements. Class I elements contain DNA sequences that are homologous to reverse transcriptase and are also known as retrotransposons (Fedoroff 1989). Transposition of class I transposable elements is mediated by an RNA intermediate that is then reverse transcribed before inserting itself into the genome, producing more than one copy of itself in the genome. The original copy of the retrotransposons is not excised from the original insertion site but is “transposed” to another site by making another copy of itself, also known as the 'copy and paste' mechanism. Retrotransposons can act as important molecular markers for detecting genomic changes associated with their activity because they create large and stable insertions in the genome (reviewed by Kalendar et al. 2011)

Class II elements, like the *Ac* and *Ds* transposons, are characterized by terminal inverted repeats (TIRs). Unlike retrotransposons, class II elements do not go through an RNA intermediate; instead, they use DNA as a direct intermediate. Transposition of class II elements requires a transposase that recognizes the TIRs at each end of the transposon (Fedoroff 1989).

The transposon system is mainly utilized to perform gene tagging. There are two major types of gene tagging: directed tagging and random tagging. For directed gene tagging, *Ac/Ds* transposons are preferred since they transpose into genomic regions close to the original site of insertion. This phenomenon has been observed and verified in *Arabidopsis*, rice, barely and many other species (Zhao et al. 2006; Singh et al. 2006; Parinov et al. 1999; Ito et al. 2002; Kolesnik et al. 2004).

Two types of transposon systems are generally used in insertional mutagenesis and gene tagging in crop plants; activator/dissociation or *Ac*/Ds element and enhancer/suppressor or mutator *(En/Spm)* elements.

2.7.1 *Ac/Ds* transposons

Ac/Ds is the two component system containing *Ds* (dissociator) and *Ac* (activator) components obtained from maize. The *Ac* element or the autonomous part encodes a transposase that binds to the terminal inverted repeat end (11 bp) of both *Ac* and *Ds*, catalyzing their transposition to new locations in the genome (Springer 2000). *Ds* elements are derivatives of *Ac* that have lost the ability to produce a transposase, but retain the terminal inverted repeats (Springer 2000). The *Ac* transposase can recognize the ends of *Ds* elements and catalyze their movement. “The maize *Ac* element is 4,565 base pairs (bp) in length and encodes an 807–amino acid transposase that catalyzes *Ac/Ds* transposition” (Zhang et al., 2006). The *Ac/Ds* element ends are delineated by complementary 11-bp terminal inverted repeat sequences. This maize *Ac* element has been expressed in *E.coli* to obtain the protein (Dylan et al., 2013).

The use of heterologous transposons in plant functional genomics began when the maize transposable element *Ac* was transformed into tobacco and was able to induce its own transposition in the tobacco genome (Baker et al., 1986). The transposon-based insertional mutagenesis approach has many advantages over other approaches for determining gene function. “Transposable elements can be mobilized or immobilized on demand and the approach requires only a few initial transformants to generate large numbers of plants carrying transposed elements (transposants or TNPs) at different locations” (Singh et al., 2006). The *Ds* element can excise from its original location and move to other places in the genome in the presence of *Ac*. Many times during transposition, footprints containing the TIR are left behind which can also induce mutations due to frame shifts. In the maize genome, the distribution of *Ds* transposons has been studied (Vollbrecht et al., 2010) for large-scale genome mutagenesis.

Ac/Ds is the most studied transposon system which can stably integrate in heterologous monocotyledon species like rice (Izawa et al., 1991; Shimamato et al., 1993), wheat (Takumi et al., 1999), and barley (Koprek et al., 2000; Cooper et al., 2004; Singh et al., 2006; Lazarow et al., 2009). An efficient strategy using *Ac/Ds* elements with the aid of tagging vectors has been described in rice (Qu et al., 2008). Using *Ac/Ds*-mediated

insertional mutagenesis, several important genes were identified in many plant species (review, Bruce and Robert, 2003). In *Arabidopsis*, these include *Curly Leaf* (Goodrich et al., 1997), and *Sporocyteless/Nozzle* (Yang et al., 1999). In maize they include *knotted1* (Hake et al., 1989) and *opaque2* (Schmidt et al., 1997), and *Branched Floretless* and *Anther Indehiscence1* in rice (Zhu et al., 2003, 2004). Recently, an activation tagging system was developed in barley based on a maize transposable element that carries two highly expressed cereal promoters (Ayliffe et al., 2009).

A two-element, *Ac/Ds*-based transposon tagging system was introduced in barley by Koprek et al. (2000) which was further improved by Singh et al. (2006). Recently, *Ac/Ds* based activation tagging (Ayliffe et al., 2007, Brown et al., 2015), a gene trap system (Lazarow et al., 2009) and insertional mutagenesis (Singh et al., 2012) in barley have been developed which has extended the role of *Ac/Ds* transposons in barley functional genomics.

2.7.2 Transposons as a tool for functional studies

The ability of transposable elements to move to a new location in the genome has made them important tools for studying gene function, particularly in plants. Insertion of a *Ds* element into a gene results in inactivation of the gene, and the resulting mutant can be used for further experimental studies (for a review on insertion mutagenesis in plants see Ramachandran and Sundaresan 2001). If the transposon moves quite frequently in the genome as is the case of *Ac/Ds* transposons, it can create large sets of insertion mutants, ideally allowing identification of a mutant in any desired gene. Insertional mutagenesis has been particularly important in maize and rice, but it has become of increasing interest in barley (Singh et al., 2012) and sorghum (Antre et al., 2013). Saturation mutagenesis was established in maize, based on the *Ac/Ds* elements and MuDr/Mu transposons (Walbot 2000; Fernandes et al., 2004).

One of the most studied insertional mutagenesis systems applied in heterologous plant species lacking efficient endogenous transposons is based on the *Ac/Ds* elements. A very efficient system for transposon mutagenesis has been developed for rice (Qu et al., 2008), and considerable efforts are being made to establish an *Ac/Ds* system in barley,

with some initial work done in wheat (Pastori et al., 2007) and sorghum (Antre et al., 2013). Barley can be relatively easily transformed by Agrobacterium mediated transformation methods and biolistics transformation. Since barley is a diploid species, insertion mutants in this crop would be highly informative for functional analysis for other Triticeae crops. Several groups have described significant progress in establishing a transposon tagging system in barley. In one study, more than 100 independent *Ds* insertions were identified and mapped; these insertions are now being used for further saturation of the genome (Zhao et al., 2006). Similarly, other studies generated a large number of single copy *Ds* insertions (Singh et al., 2006). More recently, Randhawa et al. (2009) have located single-copy *Ds* insertion events in barley using wheat cytogenetic stocks. *Ac/Ds* elements have been successfully used to study the functional genomics of malting barley QTLs (Singh et al., 2012). Other, more specific applications in barley include, gene trap approach, which allows gene identification by expression studies as well as by forward and reverse genetics (Lazarow et al., 2009). Further, an activation tagging system was developed in barley based on a modified *Ds* element fused to the maize ubiquitin promoter (Ayliffe et al., 2007; Ayliffe et al., 2009). This system should allow identification of dominant over-expression phenotypes.

2.8 Synteny among grasses

Synteny is the conserved order of genes on chromosomes of related species due to descent from a common ancestor. Many grass species such as rice, sorghum and *Brachypodium distachyon* with complete genome sequences have been proposed as models for molecular genomics in larger genome cereals like barley (Silvar et al., 2011), especially due to conservation of gene content - synteny among the Poaceae genomes (Abrouk et al., 2010). Synteny across genomes was first reported in the late 1980s between tomato and potato (Bonierbale et al., 1988). Synteny among barley and Brachypodium can offer a great perspective at chromosomal level (Ma et al., 2010). Although barley has a relatively large genome size, it has the smallest genome compared to other synteneous species such as wheat ~18000 Mbp (Gill et al., 2004), oat ~11315 Mbp (Arumuganathan and Earle 1991) and rye ~7917 Mbp (Bartoš et al., 2008). Synteneous genomes contain highly conserved order within gene structures,

which allows comparison of position and function of genes between species through comparative genomics. Hence, studies performed in other species can be applied to barley especially between synteneous regions of barley, wheat, and rice.

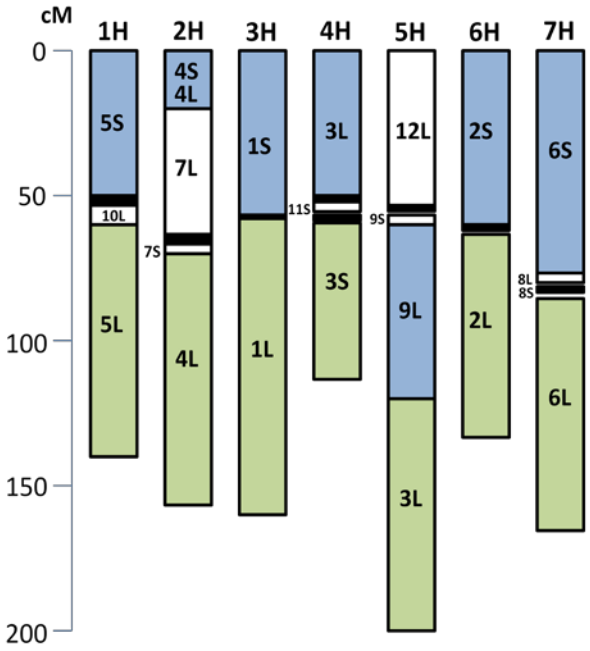
2.8.1 Barley-rice synteny

The extensive synteny across grass species is well established (e.g. Salse et al., 2008, Wang et al., 2011). Comparative mapping is a useful tool to explore small genomes of model species like rice for identifying genes in cereal crops with larger genomes such as wheat and barley (e.g. Stein et al., 2005). Comparative genomics also allows the researchers to transfer knowledge from one to the others of major crop species. Barley chromosomes have been found to be syntenic to rice in the following order: 2H–rice 4 and 7; 3H–rice 1; 4H–rice 3 and 11; 5H–rice 3, 9, and 12; 6H–rice 2; and 7H–rice 6 and 8. Figure 2.2 depicts seven barley linkage groups represented as rice synteny blocks. Numbers inside each barley chromosome indicate syntenic rice chromosome arm.

Analysis of synteny between barley and rice genomes has helped to identify specific genes in the QTL loci (Burton et al., 2006, Hazen et al., 2002 and Islamovic et al., 2013). Some important malting traits were characterized using barley-rice synteny. For instance, the centromeric region of barley chromosome 1, possessing a malting quality QTL, was found to be syntenic with rice chromosome 8 and parts of rice chromosomes 3 and 10, which aided in saturation mapping and MBC of this malting quality QTL (Han et al., 1998). Zhang et al (2011) have also shown that the terminal region of the 5HL chromosome controlling malt extract, diastatic power, alpha amylase activity is syntenic with rice chromosome 3L. On a smaller scale, micro-colinearity studies were also performed in conjunction with mapping studies to saturate small regions of the barley chromosome (Bilgic et al., 2007, Drader et al., 2009). These efforts were conducted to identify homologous genes in closely related species to aid in gene identification. The rice orthologs of the wheat *Vrn1* (Yan et al., 2003), barley *ror2* (Collins et al., 2003b), *rym4/5* (Kanyuka et al., 2005;), and *Ppd-H1* (Turner et al., 2005) are some examples of successful utilization of micro-colinearity for gene identification.

Although colinearity and micro-colinearity studies can aid greatly in identifying homologues of genes, difficulties exist in discovering genes from non-linear genomic regions. Hence, in some instances, synteny and micro-colinearity can provide only limited insight into genome organization and alternative methods have to be utilized to understand genome organization and gene function in barley.

Figure 2.2: Synteny between barley and rice, showing seven barley chromosome (1H to 7H), on scale of 1 to 200cM with their corresponding rice syntenic region inside the chromosome blocks.



2.9 Barley malting

A characteristic of barley which makes it suitable for beer production is its malting quality. Malting involves many enzymes that have a collective effect on beer quality. The malting process involves three stages: steeping, germination, and kilning. During steeping, barley seed is soaked in water to reach predetermined moisture content. During the germination phase, hydrolytic enzymes are synthesized by the aleuronic cells (Bamforth and Barclay 1993) and scutellum (Ranki 1990). These hydrolytic enzymes are secreted into the starchy endosperm where they convert proteins and carbohydrates into partially degraded biopolymers. Kilning involves air drying of the malted barley at 40–60°C and the temperature is gradually increased to 85–95°C. Other than the low molecular weight biopolymers, malt also contains heat-stable hydrolytic enzymes required for further breakdown of carbohydrates in the subsequent mashing process.

2.10 Genetics of barley malting quality

Malting quality is a complex phenotype that combines a large number of interrelated components, each of which has complex inheritance (Hayes and Jones 2000). Enhancement of malting barley cultivars involves the improvement of as many as 22 malting quality traits such as, enzyme activity, kernel plumpness, malt extract yield for starch modification and percent grain protein, along with many agronomic traits including grain yield, lodging resistance and seed shattering (Rasmusson and Phillips 1997).

Genetic studies of malting quality phenotypes resulted in more than 250 QTLs associated with 19 malting quality traits (Gao et al., 2004; Wei et al., 2009). Recently, Islamovic et al., (2014) identified additional QTLs on chromosome 1H, 4H, 5H, 6H and 7H linked with malting traits like malt extract (ME), wort protein, soluble/total protein (S/T), diastatic power (DP), alpha-amylase, β-glucan (BG) and free amino nitrogen (FAN) using Stellar/01Ab8219 mapping population.

About 84% of malting quality QTLs are conserved among different mapping populations. Through the efforts of the North American Barley Genome Project

(NABGP), several QTLs of agronomic and industrial importance were mapped on chromosome 4H (Hoffman et al., 2002; Mather et al., 1997; Hayes et al., 1993). Four major QTLs on chromosome 4H affecting various malting traits were mapped by Marquez-Cedillo et al., 1999. A major QTL detected in Steptoe X Morex population at ABG003a-MWGO58H was shown to have a large effect on Test Weight (TW), Grain Protein (GP), ST (soluble total protein) and AA (α-amylase), with 27% phenotypic variation. Other QTL affecting traits like ME (Malt Extract), TW and AA, mapped in int-c-HVM40H interval, show a phenotypic variation of 38%. In addition, two smaller QTLs affecting KP (Kernal plumness) and DP (Diastatic power) with 32% and 26% respective phenotypic variation were also detected. Further improvements in mapping strategies, led to the identification of malting quality traits in the 4H telomeric region demarcated by MWG634 and CDO669 loci (Gao et al., 2004). This region is identified as QTL2 (0 to 15.8 cM), which affects several malting quality parameters, namely, ME, DP, AA, BG and Dormancy (also shown by Hayes et al., 1993).

Malt quality is controlled by the action of many different starch and protein degrading enzymes. A perfect blend of modified and non-modified starch and protein levels are required to brew beer of acceptable quality. Some genes associated with improved malting quality have been identified. α-amylase I (E.C. 3.2.1.1), which cleaves the α-(1-4) glucosidic bonds of starch, is produced only during initial germination stages under the control of the *Amy2* gene. A second form of α-amylase; α-amylase II (*Amy1)* becomes highly active in the mashing stage and modifies the remaining starch. Another key enzyme, β-amylase (E.C. 3.2.1.2), is involved in the production of the fermentable sugar maltose, which is utilized by yeast during fermentation. β-amylase is a multi-geneic locus (*Bamy1, Bamy3*) (Li et al., 2002), and can catalyze the hydrolysis of ~70% of amylose and ~50% of the amylopectin fraction of starch. However, it is highly thermostable and remains active until the mash temperature exceeds 55^0C. Another important enzyme; β-glucanase, has received recent attention due to its β-glucan hydrolysis activity. High amounts of unmodified β-glucan lead to negative impacts on beer filterability and hot water extract (Stewart et al., 2000). Due to the loss of β-glucanase activity above 45^0C, β-glucan hydrolysis in malting remains an important

target in barley breeding programs. Limit dextrinase (E.C. 3.2.1.41) catalyzes the hydrolysis of α-(1-6) glucosidic linkages of amylopectin. *Ldx* genes are expressed during germination and have a positive effect on wort fermentability.

Whereas certain genes associated with improved malting quality have been identified, large number of QTLs associated with malting quality traits suggests that many other genes play important roles in malting, suggesting need for further investigation. In spite of many efforts to saturate the QTL2 with molecular markers, the gene/genes present in this important malting QTL have not been characterized. Therefore, the main focus of this study is to dissect the QTL2 malting region using various molecular and genomic tools.

2.11 β-glucan content: An important malting trait

Among various traits that affect malting quality (i.e., malt extract content, α- and β-amylase activity, diastatic power, malt β-glucan content, malt β -glucanase activity, grain protein content, kernel plumpness, and dormancy), the amount of β-glucan present in germinating and malted barley plays a crucial role in producing good quality beer.

β-glucans are complex mixed linkage polymers composed of (1→3), (1→4) linked β -D-glucose units. They comprise an important component found in the cell walls of barley and other cereal grains. They are nonstarchy polysaccharides found in the aleurone cell layer and in the cell walls of the endosperm. They constitute about 75% of all carbohydrates and one of the most studied homopolysaccharides involved in brewing (Izydorczyk and Dexter, 2008).

β-glucans have been recognized as causes of brewing problems such as poor mash conversion, retarded and inefficient wort and beer filtration and nonbacterial colloidal hazes in the final product (Bamforth 1982).

Gjertsen (1966), of the Carlsberg Breweries, Denmark, was the first scientist to identify the chemical composition of a gelatinous material which was causing processing problems when brewers attempted to filter beers. In his findings he was the first to characterize the precipitate as a mixture of (1→3), (1→4)- β-glucan with (1→4)- β –

linkages. Further, in 1977, *The Practical Brewer* recognized that excess β -glucans in barley may lead to problems in the malting process and increased wort viscosity causing slow lautering and filtration (Adamic 1977; and Meilgaard 1977).The structure of β-glucan was demonstrated by Fleming and Kawakami (1977) by extracting β–glucans from barley at different temperatures. They concluded that typical β -glucan molecule is composed of glucose monomers linked, 30% of the time with β -(1→3)-glycosidic bonds and the rest of the time with β -(1→4)-glycosidic bonds. Hwang and Lorenz (1986) examined 18 cultivars of barley grown in 10 different harvest sites in the United States and Canada and found that the amount of β-glucan content appeared to be more influenced by the growing conditions of barley than it was by its cultivar characteristics.

The endogenous hydrolytic enzymes which degrade the endosperm cell walls of barley during malting were further identified and characterized. Many studies were conducted to extract β-glucan degrading enzymes like β-gluconase (Manners and Wilson 1976, Woodward and Fincher 1983) at different stages of malting and brewing, but none of them suggested that β-glucans are completely hydrolysed by these enzymes. Rather, the end products are β-linked tri-, tetra-, or oligosaccharides.

β-gluconase enzymes were reported to be present in very low quantity in barley grains, and their content dramatically increased after malting (Wang et al., 2004). However, the endogenous barley β-glucanases synthesized during germination are damaged during mashing temperature of 50^0C. As a result of these factors, the content of β-glucans in wort increases (Jin et al., 2004).

2.12 Thaumatin like proteins (TLPs) and β-glucan

Higher plants employ a number of defenses against infections by pathogens, pests, and abiotic stresses. Among the various protective adaptations is the synthesis of pathogenesis-related (PR) proteins. PR protein in the plants was first discovered in tobacco plants infected by tobacco mosaic virus (Van Loona 1970). Many thaumatin-like proteins (TLP) have been shown to be antifungal in *in-vitro* assays (Hejgaard et al.,

1991, Yun et al., 1997) as well as in transgenic plants (Anand et al., 2003, Kalpana et al., 2006)

Liu et al (2010) reviewed the superfamily of TLPs, its origin, evolution, and biological function. PR proteins have been classically divided into five families based on molecular mass, isoelectric point, localization and biological activity. The PR5 class includes a group of TLPs expressed in leaves, including osmotin of tobacco (Singh et al., 1987), PRHv-1 of barley (Hahn et al. 1993), PWIR2 of wheat (Rebmann et al., 1991) and RASTLs of oat (Lin et al., 1996). Thaumatin, an intensely sweet-tasting protein, originates from the African shrub *Thaumatococcus daniellii*. TLPs contain 16 cysteine residues which are involved in the formation of eight disulfide bridges.

Barley contains eight thaumatin-like proteins, TLP1-TLP8 with a broad range of optimum pH range, from acidic (TLP1-TLP3) to weakly acidic (TLP4) to basic (TLP5-TLP8). The apparent molecular masses range from 15 to 24 kDa (Reiss 2001). Information about the barley TLPs 1-8 protein is presented in Table 2.1.

Isoforms of PR-5 proteins provide antimicrobial and antifungal activity in malted barley (Gorjanović et al., 2007). PR-5 proteins have also been suggested to bind β-glucans, and, therefore, might have carbohydrate-binding properties (Trudel et al., 1998; Osmond et al., 2001). Barley TLPs have a high degree of protein-polysaccharide-binding specificity with β-glucan. Osmond et al (2001) and Trudel (1998), indicated that TLPs bind only water-insoluble β-1,3-glucans such as pachyman, curdlan, paramylon, zymosan, alkali-insoluble bakers' yeast (*Saccharomyces cerevisiae*), and *Pleurotus ostreatus* glucan, but not chitin, pustulan or cellulose. Furthermore, higher binding was observed for linear (1,3)- β -d-glucans like debranched pachyman and boiled curdlan. Kinetics of the binding interactions suggested the optimum pH was 5.0 and binding was lost by boiling in the presence of SDS (Osmond et al. 2001).

Molecular modelling of TLPs, tobacco PR5d and zeamatin, suggest that these TLPs are globular proteins, consisting of a flattened β - barrel adjacent to a more flexible region rich in loops and coils that are stabilized by disulfide bonds with a large negatively charged acidic cleft. Systematic searches of the TLP models revealed regions on the

surface of the model that might interact with a (1,3)-β glucan. Docking of linear (1,3)- β -D-glucan in the cleft of barley TLP, reflected in the density of isoenergy contours, suggests that the polysaccharide-binding site is likely to be located in this cleft.

Table 2.1: Data presented from the sequences of the cloned cDNAs of barley TLPs (adapted from Reiss et al. 2006)

	GenBank locus	Molecular mass (Da)	Theoretical pI	Mature peptide (aa)	Cysteine residues
TLP1	AY839292	15626	4.33	153	10
TLP2	AY839293	15654	4.51	153	10
TLP3	AY839294	16071	4.85	153	10
TLP4	AF355455	15873	5.71	149	10
TLP5	AY839295	22724	6.24	213	16
TLP6	AF355456	21352	7.53	202	16
TLP7	AF355457	21379	7.91	203	16
TLP8	AF355458	21855	8.15	208	16

CONNECTING STATEMENT BETWEEN CHAPTER II & III

In chapter II, I reviewed the current status of the barley genome, gene identification, mapping strategies, comparative genomics and malting quality traits. Based on my critical literature review, I observed that malting quality is an important aspect of the barley breeding industry and QTL2 is one of the major QTLs influencing traits like malt extract, diastatic power, β-glucan and dormancy. As in our laboratory, material and expertise especially for transposon mutagenesis were available; I took the initiative to use these available resources to understand this important chromosomal region of barley.

Chapter III highlights the dissection of two major malting quality QTLs located on chromosome 4H of barley using the *Ac/Ds* transposon system. From a repository of stable *Ds* insertion lines (TNPs), TNP-29 and -79 were selected as they are located in the vicinity of the malting quality QTLs on chromosome 4H. Reactivation of these lines was performed using conventional breeding and tissue culture methods. Since the *Ds* transposon tends to re-insert near the original site of insertion, generation of new *Ds* insertion lines can be utilized for the saturation of the malting quality QTLs on chromosome 4H. We reported a threefold increase in reactivation frequency through the *in vitro* approach. The results in this section were published in Functional & Integrative Genomics (2012, Vol 12, pp. 131-141)

I contributed to the following chapter by designing the experiment, conducting most of the work and writing the manuscript. Funding for this project was provided by the Brewing and Malting Barley Research Institute and NSERC through Dr. Jaswinder Singh.

Chapter III

Mutagenesis of barley malting quality QTLs with *Ds* transposons

Surinder Singh, Han Qi Tan and Jaswinder Singh*

*Corresponding Author: Dr. Jaswinder Singh

Plant Science Department, 21 111 Rue lakeshore

McGill University, Quebec, H9X 3V9, Canada

Email: jaswinder.singh@mcgill.ca

Phone: +1(514) 398-7906

Reprinted with permission:

Singh S, Tan H-Q, and Singh J (2012) Mutagenesis of barley malting quality QTLs with *Ds* transposons. **Functional & Integrative Genomics** 12:131-141

3.1 Abstract

Various functional genomic tools are being used to identify and characterize genes in plants. The *Ac/Ds (Activator/Dissociation)* transposon-based approach offers great potential, especially in barley, due to limited success of genetic transformation and its large genome size. The bias of the *Ac/Ds* system towards genic regions and its tendency toward localized transpositions can greatly enhance the discovery and tagging of genes linked to *Ds*. Barley is a key ingredient for the malting and brewing industry, therefore gene discovery in relation to malting has industrial applications. Malting quality in barley is a complex and quantitatively inherited trait. Two major QTLs, affecting malting quality have been located on chromosome 4H. In this study, *Ds* was reactivated from parent transposants (TNP) lines,TNP-29 and TNP-79, where *Ds* was mapped in the vicinity of important malting QTLs. Reactivation of *Ds* was carried out both by conventional breeding and *in vitro* approaches. A threefold increase in reactivation frequency through the *in vitro* approach enabled the development of a new genomic resource for the dissection of malting QTL and gene discovery in barley. Identification of unique flanking sequences, using High Efficiency Thermal Asymmetric Interlaced) (HE-TAIL PCR) and inverse PCR (iPCR) from these populations, has further emphasized the new location of *Ds* in the barley genome and provided new transposon mutants especially in *β-GAL1*, *β-amylase* like gene and *ABC transporter* for functional genomics studies.

Keywords: *Ac*/*Ds*, Transposon reactivation, Genomics, Transformation, Barley, Cereals.

3.2 Introduction

Genome sequencing is generating enormous amounts of data which challenge the researchers to elucidate their role in the genome. Mutagenesis, via T-DNA insertion, RNA interference (RNAi) and transposon tagging enables one to investigate the function of unknown but important genomic sequences (Alonso and Ecker 2006; Li et al., 2011). Map-based cloning has also been employed for gene identification in various plant species (Keller et al., 2005; Watanabe et al., 2009, 2011). However, these methods can be inefficient, especially in genomes with large and repetitive sequences. For example, in barley and wheat, chromosome walking is inherently difficult and inefficient (Peters et al., 2003; Lee et al., 2007). Another drawback of map-based cloning is in identifying tightly linked molecular markers with gene of interest. The transposon-based insertional mutagenesis approach has certain advantages over other approaches in determining gene function, especially where genetic transformation is not routine. A transposon-based approach requires only a few initial transformants to generate large numbers of plants carrying transposed elements (transposants) at different locations (Singh et al., 2006).

The use of transposons for plant functional genomics in heterologous species was first reported with the introduction of a maize transposable element *Ac* (*Ac*tivator) in tobacco (Baker et al., 1986). The *Ac/Ds* system is the most widely used transposon system for mutagenesis in plants. This particular family comprises an autonomous *Ac* element, encoding the 807-amino acid transposase protein (*Ac*TPase) that catalyzes the transposition of both *Ac* and *Ds* elements (non-autonomous element) (Kunze and Starlinger, 1989). *Ds* lacks transposase, while retaining the sequences critical for its transposition – namely 11-bp terminal inverted repeats (TIRs) and subterminal regions of 250 to 300 nucleotides on both ends (Coupland et al., 1988; Varagona et al., 1990). While *Ac* contains the crucial *Ac*TPase transposase enzyme, the *Ds* encodes a mutated or nonfunctional transposase.

By using an *Ac/Ds* tagging system, several important genes have been identified (Ayliffe et al., 2009; Jiang and Ramachandaran, 2010). Most were isolated using an undirected tagging approach in which large populations of transposon-containing plants

were generated and screened for phenotypic abnormalities. Taking advantage of the tendency of *Ac/Ds* to transpose locally (Smith et al., 1996; Parinov et al., 1999; Upadhyaya et al. 2002), a *Ds* element can be placed near a gene of interest, which can then be tagged by localized mutagenesis (Upadhyaya et al., 2006). Examples demonstrating the power of this localized tagging approach in maize include the cloning of *tasselseed2* (DeLong et al., 1993), *indeterminate1* (Colasanti et al., 1998), and *pink scutellum1/viviparous7* (Singh et al., 2003).

Barley is a significant industrial crop with growing roles in food and bioproducts, but it is most commonly used for feed, and as malt in the brewing and distilling industry. It is also an important model plant for genetics research as it is a true diploid with high homology to other economically important *Triticeae* crops, especially wheat. Barley has a large genome comprising about 5.6×10^9 bp. About 80% of the genome consists of highly repetitive DNA, mostly consisting of retrotransposons (Sreenivasulu et al., 2008). In spite of the fact that barley has a huge genome size, it remains the smallest genome compared to other species sharing close synteny, such as wheat (~5500MB–18000MB) (Gill et al., 2004) and oats (11315MB) (Arumuganathan and Earle, 1991). Genetic resources have expanded exponentially in barley: They include high density maps (Close et al., 2009; Schulte et al., 2009), ESTs, microarray (Close et al., 2004) and TILLING lines (Caldwell et al., 2004; Talame` et al., 2008).

The feasibility of transient *Ac* expression to transpose *Ds* has been documented in different plant species (McElory et al., 1997; Weld et al., 2002; Upadhyaya et al., 2006). The transposition of *Ds* elements in barley was first demonstrated when McElory et al., (1997) used a simple transient assay in which *Ac*TPase was introduced simultaneously with the *uid*A (*β*-glucuronidase) gene disrupted by *Ds.* They demonstrated the excision of the maize *Ds* transposons in scutellar tissue. Using stable transformation methods, *Ds* lines were created which developed into a valuable resource for the saturation mutagenesis of linked genes. Amongst them are the single copy *Ds* insertion barley lines developed by Singh et al (2006). Two of these *Ds* loci in lines TNP-29 (Cooper et al., 2004) and TNP-79 (Randhawa et al., 2009) are located in the vicinity of two important malting quality QTLs on chromosome 4H, and therefore are excellent

candidates to saturate malting quality QTL regions with *Ds* transposons. The *Ds* element in TNP-29 is located 10cM from QTL2 on the short arm of chromosome 4H, which greatly affects malting quality parameters (Hayes et al., 1993; Zale et al., 2000; Gao et al., 2004). Because the *Ds* transposons tend to re-insert into genic regions that are closely linked to the original site of excision (Smith et al., 1996; Parinov et al., 1999; Koprek et al., 2000; Greco et al., 2003; Upadhyaya et al., 2002), the reactivation of *Ds* from these launching pads can facilitate saturation mutagenesis. In the present study, a single copy *Ds* element from TNP-29 and TNP-79 was exploited as a launch pad for its reactivation. This was attained through hybridization with the *Ac*TPase expressing line and via the transient expression of *Ac*TPase in immature embryos using *Agrobacterium tumefaciens*. A new source population has been developed to facilitate the cloning of these important malting-quality QTL regions. In the process new transpositions were identified and flanking sequences generated and characterized through bioinformatic approaches.

3.3 Materials and Method

3.3.1 Genetic stock

For the development of new TNP lines, seeds of barley lines TNP-29, TNP-79 and 25-B, were obtained from the TNP repository at USDA, Aberdeen, Idaho. The TNP-29 and TNP-79 lines are single copy *Ds* insertion lines, whereas 25-B is the *Ac* transposase expressing line (Singh et al., 2006).

3.3.2 Generation of new *Ds* lines

For the conventional approach, seeds were planted in a greenhouse under a 16h light/8h dark cycle with a day temperature of 18°C and a night temperature of 15°C, and 50% relative humidity with a light intensity of 1000 µE. Reactivation of *Ds* in TNP-29 and TNP-79 lines was carried out separately by hybridizing them with 25-B. Seeds of TNP-29X (TNP-29 X 25-B) and TNP-79X (TNP-79 X 25-B) were collected, grown and allowed to self-pollinate in order to obtain F_1 and F_2 generation seeds for further analysis.

For the *in vitro* approach, seeds of TNP-29 and TNP-79 were planted in a phytotron under conditions of 70% relative humidity, a 16 h photoperiod at 18°C during the day and at 15°C during night. Spikes were collected 14 days post anthesis, surface sterilized with 70% ethanol, and immature embryos were excised for use as an explant for *Agrobacterium* transformation with a construct containing the *Ac*TPase gene.

3.3.3 Generation of *Ac*TPase construct

The pCambia-Act*GFP*-UbiAc construct contained the *Ac*TPase gene under the control of an Ubiquitin promoter (pUbiAc), and a *GFP* gene driven by an actin promoter (pAct*GFP*) in a pCambia 1300 binary vector. Both *Ac*TPase and *GFP* ORFs were fused to a nopaline synthase (nos) terminator within the left and right borders of the same plasmid (Figure 3.1). The construct was created in two steps. First, pUbiAc plasmid was digested with a *Pst*I restriction enzyme to obtain a UbiAcTPase fragment and ligated into a pCambia 1300 binary vector. Second, the digestion of pAct*GFP* plasmid with *Kpn*I and *Bam*HI enzymes released Act*GFP* fragments, which were purified and ligated into pCambia-UbiAc.

3.3.4 Agrobacterium-mediated transformation

Embryo transformation using *Agrobacterium tumefaciens* was carried out with the protocol developed by Gurel et al (2009). Immature embryos were incubated in co-cultivation (CO) medium to induce *Agrobacterium* transformation. They were then transferred to solid CO medium to enhance *Agrobacterium* transformation and then to a callus induction medium (CI). Transformed tissues were selected using 50 mg/L hygromycin and 300 mg/L timentin in CI media. Embryos were incubated in the dark on the CI medium for further screening. To detect GFP fluorescence, embryos were observed using a Nikon SMZ1500 fluorescence stereomicroscope with a GFP-1 filter at an excitation wavelength of 480/40 nm. Transient expression was scored at 3, 10 and 17 dpi (day's post-inoculation) with *A. tumefaciens*; the embryos expressing GFP were separated from non-GFP expressing ones.

After 2 weeks of sub-culturing on modified CI media calli were regenerated and transferred onto rooting transition (RT) medium for shoot induction. Shoots that emerged were shifted onto rooting (R) medium. Seeds were collected and planted in the greenhouse to yield further generations.

3.3.5 Molecular screening of *Ds* elements

A *Ds* construct with the bar gene (which provides resistance against glufosinate ammonium IGNITE®, Bayer CropScience, Canada), was used for primary screening of plants containing the *Ds* element after crossing with *Ac*TPase-expressing plants. The herbicide, IGNITE in a concentration of 0.33% was applied to the young leaves of the plants at the 3-4 leaf stage and leaves were examined for symptoms of necrosis after 5 days. Plants containing a *Ds* element remained healthy after herbicide treatment and were selected for molecular analysis. Identification of *Ds* transposition was conducted by PCR based molecular screening of F_2 populations in both the TNP-29X and TNP-79X lines. DNA extraction was carried out using young leaves with the standard phenol: chloroform based protocol described by Singh et al. (2006).

Progenies containing *Ds* insertions and *Ac*TPase, were identified using primer pairs, JNOSF & JDS-3R, and AC3 & AC5, respectively. The movement of *Ds* to a new location was confirmed using *Ds* flanking primers; JIPF1 & JPT293R for TNP-29X and JIPF2 & JPT793R for TNP-79X lines. PCR reaction contained 50 ng of genomic DNA, 2.5µl of 10X PCR buffer, 2.5 µM of dNTPs, 0.5 µM, of each primer (forward and reverse) of 1% DMSO, 2.5 U of *Taq* DNA polymerase (GoTaq Promega, USA) and ddH_2O to make a final volume of 25 µl. PCR was performed using the GeneAmp PCR 9700 System (Applied Biosystems, USA) with an initial denaturation at 95°C for 2 min, followed by 36 cycles consisting of 95°C for 30 s, 60°C for 45 s, and 72°C for 60 s.

Regenerated plants were screened for *Ds* transpositions. PCR analysis was conducted for an empty donor, an original flanking sequence, and for *Ds* and *Ac* sequences .The empty donor PCR was performed with primers JPT295R and JPT293R for TNP-29; JPT795R and JPT793R specific for TNP-79 designed from the flanking regions on both

sides of the original insertion sites of *Ds*. PCR was conducted as described in previous section. PCR primer pairs are listed in Table 3.1.

3.3.6 Isolation of *Ds* flanking sequences

Sequences of *Ds* flanking regions from new transpositions were obtained through the HE-TAIL PCR (Tan and Singh 2011) and iPCR. The HE-TAIL PCR was performed in four separate runs using genomic DNA, Takara ExTaq DNA polymerase, 10X PCR buffer containing 10 mM $MgCl_2$, and 2.5 mM dNTPs (Takara-Bio, Dalian, China). These PCR runs include pre-amplification, primary amplification, secondary amplification and tertiary amplification, respectively, following the conditions described by Tan and Singh (2011), using GeneAmp® PCR System 9700 (Applied Biosystems, USA).

For iPCR, genomic DNA was digested with either *Nco*I or *Nhe*I (both enzymes have a restriction site in the *Ds* element), followed by heat inactivation and phenol:chloroform purification of the digested DNA. The digested DNA was self ligated and the product was used for subsequent PCR. The cocktail for the first cycle of the iPCR was prepared by adding 1.0 µl of 0.1 µg of purified and ligated DNA, 2.5 µl of 10X buffer, 2.5 µl of 2.5 mM dNTP mixture, 0.5 µl of 10 µM of the first set of nested primers for the 5' end JIPR3and JIPF4 and the 3'end JIPR9 and JIPF2 and 0.25µl of 1U of Ex Taq DNA polymerase (Invitrogen, USA) in a total reaction volume of 25µl. PCR products from the first round were then diluted to 20X in the 5' end and 50X in the 3' end with water and used as template for the second round of PCR. Specific PCR products were generated in the second round of PCR by amplifying with nested primers JIPR5 and JIPF7 on 5´ side and JIPR6 and JIPF1 on 3´ end. The products obtained from second iPCR were gel purified using gel extraction kit (QIAquick® Gel extraction kit, Qiagen, USA).

3.3.7 Bioinformatic analysis

The amplified fragments from the TAIL PCR and iPCR were ligated into a TOPO-TA-cloning vector (Invitrogen, USA) and sequenced. Bioinformatic analysis of these flanking sequences was carried out using a BLAST algorithm (Basic Local Alignment Search Tool). EST's and similarly expressed proteins related to all the 5' and 3' flanking sequences were queried against the EST database HarvEST (http://harvest.ucr.edu) (Close et al., 2007), NCBI (http://www.ncbi.nlm.nih.gov/) and Gramene (http://www.gramene.org). Flanking sequence and ESTalignment were performed with software clone manager 9. GenScan (http://genes.mit.edu/) (Burge and Karlin 1997) was used to predict coding sequences. For describing gene product characteristics and gene product annotation, GO (Gene Ontology) analysis was also carried out (http://www.geneontology.org/) (Ashburner et al., 2000)

3.4 Results

3.4.1 Generation of new *Ds* insertion lines

A total of 593 plants from TNP-29X and TNP-79X populations were screened. As the *Ds* construct contains the *bar* gene, the plants showing resistance to IGNITE herbicide painting were selected in the F_2 generation. Selected plants were screened by PCR analysis and Southern blot analysis to confirm the presence of *Ds*.

For *in vitro* experiments, 365 embryos from TNP-29 and TNP-79 were used for transformation. A total of 143 embryos from TNP-29 were transformed and 46 plantlets were recovered. For TNP-79, 222 embryos were transformed and 64 plantlets were regenerated. A total of 110 embryos were successfully regenerated into shoots. However, multiple shoots were obtained for some embryos which resulted in the production of 116 plantlets. The frequency of transformed embryos expressing *GFP* was examined on the 3rd, 10th and 17th dpi and was found to be 49.6%, 16.7% and 7.9%, respectively. A negative correlation between *GFP* expression and number of dpi was also observed. *GFP* expression decreased from a frequency of 49.6 % on the 3rd dpi to 7.9% on 17th dpi (Figure 3.2). We also observed a positive correlation between

the size of *GFP* spots in transfected tissues and the dpi. This expression pattern indicated the presence of stable *Ac*TPase transformation.

3.4.2 Molecular screening of *Ds* elements

Three different PCRs were performed (Figure 3.3) to analyze the transposition of *Ds*. Figure 3.3a shows the presence of *Ds* in its original location. To identify a new *Ds* insertion, lines with positive *Ds* transposon amplification and negative amplification of the flanking sequence were selected. Figure 3.3b depicts flanking sequence PCR. Furthermore, to separate stable (absence of *Ac*TPase) and unstable *Ds* lines, the presence of *Ac*TPase was determined by PCR (Figure 3.3c). Lines containing *Ds* at a new position but without *Ac*TPase were considered as new stable *Ds* insertions. In TNP-29X, out of 370 lines screened, 39 showed new transpositions. Among these 17 were stable (*Ac* absent) and 22 were unstable (*Ac* present). In TNP-79X, out of 223, there were 22 new *Ds* transpositions; 13 stable and 9 unstable. New *Ds* transpositions observed in TNP-29X and TNP-79X populations were 10.5% and 9.8% respectively (Table 3.2). In addition to PCR analysis, confirmation of new *Ds* transposition events was carried out by Southern blot analysis (data not shown).

Similarly, in plants generated through tissue culture, PCR based analysis (Figure 3.4) confirmed the presence and transposition of *Ds* elements and presence of *Ac*TPase in the regenerated plants. Figure 3.4(a) shows results of flanking PCR to determine the presence or absence of *Ds* at its original position in TNP-29. Samples showing negative amplification (*Ds* having jumped to a new location) were further selected to confirm the presence of *Ds* in the genome (Figure 3.4b). For example, samples number 1, 2, 3, 8 and 9 *Ds* have relocated in TNP-29. Similarly, figure 3.4(c) and 3.4(d) depict movement of *Ds* to new location in TNP-79 (samples 3, 6 and 9). To reconfirm the *Ds* transposition, further analyses were conducted by empty donor PCR and southern blotting (data not shown). Stable transpositions (Table 3.2) were observed in both TNP-29 (11 of 46) and TNP-79 (22 of 64). *Ds* transpositions were observed in both *GFP* and non-*GFP*-expressing embryos; nonetheless the transposition frequency was higher in *GFP* expressing embryos. Results clearly indicate the reactivation of the *Ds* element

through *in vitro* expression of *Ac*TPase. In the T_0 population, the total percentage of *Ds* transpositions occurring after the transient expression of *Ac*TPase was 34.7% for TNP-29 and 39.06% for TNP-79. These frequency rates were calculated on the total number of new *Ds* transpositions regardless of *GFP* expression against the total number of regenerated plantlets for each line. Newly generated transposants identified in T_0 generation were further investigated for the presence of *Ac*TPase in order to obtain stable transposants. In the case of TNP-29, approximately 10% of transformants (5/46) show the presence of *Ac*TPase. However, only 4% (3/64) of plants containing *Ac*TPase were found in TNP-79. The plants containing *Ac*TPase were subsequently analyzed in the T_1 generation for the integration of this gene. *Ac*TPase was only detected in some T_0 plants but not in T_1 plants (data not shown), indicating that *Ac*TPase was not integrated in the genome, and *Ds* was activated by the extra chromosomal expression of *Ac*TPase. *Ds* transpositions generated through tissue culture and conventional methods were compared which clearly demonstrated enhanced *Ds* activity with transient expression of *Ac*TPase.

3.4.3 Characterization of flanking sequences

Some of the new *Ds* transpositions were subjected to HE-TAIL PCR and iPCR to obtain flanking sequences. Based on a BLAST search, the majority of TNP flanking sequences matched known ESTs or characterized gene sequences (Table 3.3). Analysis of these flanking sequences with the gene prediction program also envisaged the majority of transpositions to be in genic regions (Figure 3.5). Further investigation of flanking sequences from TNPs indicates that the insertion sites of two TNP lines had damaged TIRs (Table 3.3). Several important genes were tagged by *Ds* including the *β-GAL1*, the *β-amylase* like gene, and the *ABC transporter.* Sequence analysis showed the presence of intact terminal inverted repeats (TIRs) in the majority of flanking sequences; flanking sequences from MTNP-201, MTNP-204 and MTNP-208 indicate that the insertion sites of these TNP lines have damaged TIRs. MTNP-201 has a 2 bps deletion from the 3' end; MTNP-204 has a 2 bp G to C transversion, and MTNP-208 has the last 2 bp deleted from the 3' end. This analysis (Table 3.3) also confirms the presence of *Ds* in

new locations as all these sequences were observed in different genic regions from the parental *Ds* lines (TNP-29 and TNP-79).

A partial ORF encoding a chromodomain, containing protein, was predicted in the flanking sequence of the MTNP-201 line. Further, analysis of corresponding ESTs indicated expression in germinating seed at the malting stage (16-48 h). Also, the flanking sequence from MTNP-210 shows similarity with the chromodomain protein except for a shorter exon region. ESTs (Genbank: DN180650) showed expression in leaf (heading stage), seeds, caryopsis and spike. A predicted ORF of MTNP-202 sequence contained an ATP binding cassette. Corresponding ESTs obtained showed its expression in endosperm at the malting stage (Unigene no.8212) and in epidermis at the seedling stage (Genbank No. CD056233). The MTNP-203 sequence has a predicted coding region of 150 bp which corresponds to EST expressed in seed, embryo and scutellum at various stages of malting (0-16 h and16-48 h germination stage), and in seedling, spike and root (Genbank No. BY845649, Unigene: 530 in HarvEST database). The mapping location of this EST was found on the 4H chromosome (Hedley 2001 unpublished; HarvEST: Barley database). BLASTP analysis of this flanking sequence revealed it to be a, glutamine synthetase. The MTNP-204 sequence has a small region predicted to encode a β-amylase-like gene, which is known to be involved in malting quality in barleyand EST information suggests its expression at the germinating seed stage (Genbank no. CA019624). BLAST analysis of MTNP-206 showed partial similarity with the F-domain box containing the predicted protein and also showed partial similarity with the *β-GAL1* gene, which is thought to play an important role in malting and seed germination. Corresponding ESTs also expressed in seeds at the malting stage of 0-16 h germination (Genbank No. CA007240). MTNP-207 had a small region encoding a fibre protein expressed in the plasma membrane. MTNP-209 did not match any coding region and BLAST analysis showed no significant match.

Figure 3.1 Partial map of the pCAMBIA-UbiAcTPase-ActGFP construct, indicating the orientation of *Ac*TPase and *GFP* on the vector.

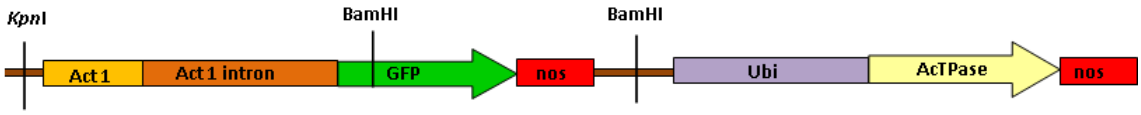


Figure 3.2 The use of a GFP visual marker (encircled in red) in embryos of TNP-79 to identify transformed tissue [3rd (left) and 17th (right) dpi].

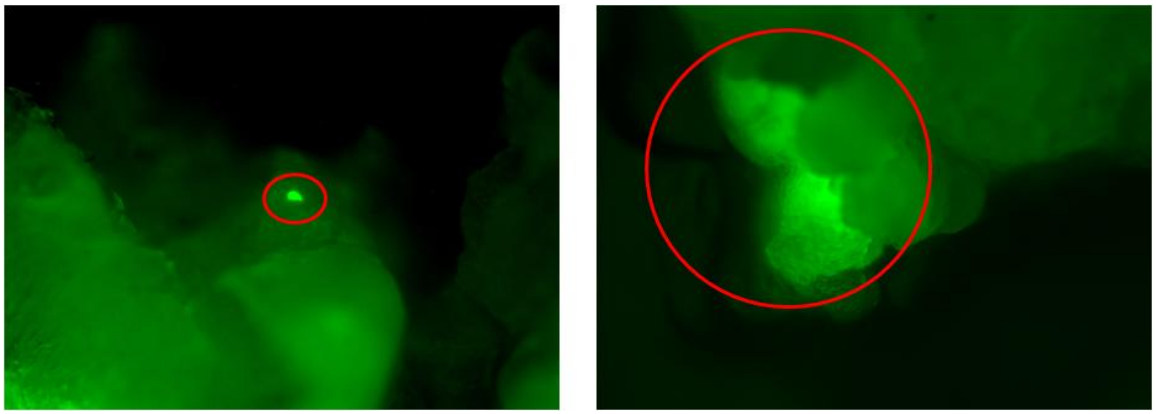


Figure 3.3 Molecular screening of *Ac/Ds* elements in TNP-29X and TNP-79X a) Positive amplification indicates the presence of *Ds*. b) PCR analysis to score *Ds* movement (amplification indicates *Ds* in its original position whereas negative amplification indicates the transposition of *Ds* to a new location). c) Amplification indicates the presence of *Ac*. M represents a molecular weight marker. H_2O and GP (Golden Promise-non transgenic barley), negative controls. TNP-29, positive control for *Ds* and Flanking PCR; 25-B as positive control for *Ac* PCR and; p*Ds* and p*Ac*, positive controls for *Ds* and *Ac* respectively.

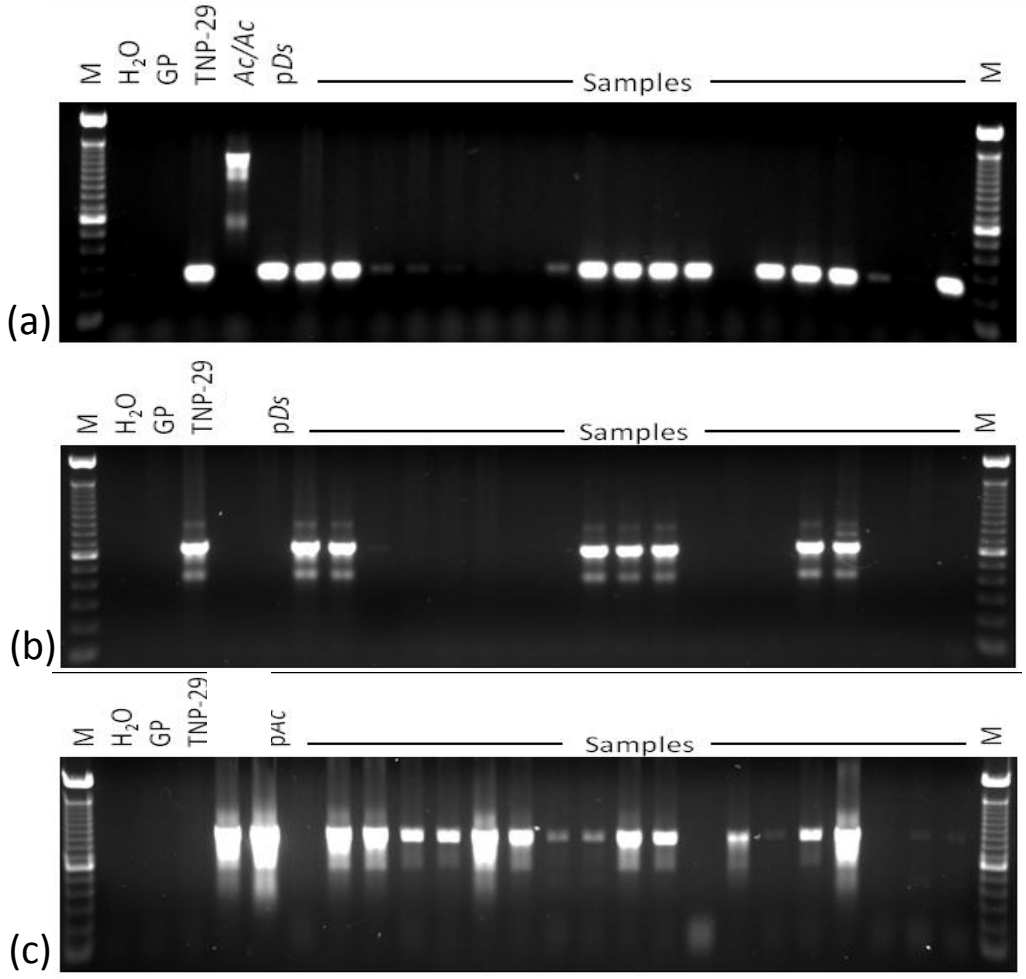


Figure 3.4 PCR analysis of *Ac/Ds* elements in regenerated plantlets through tissue culture a) Original flanking sequence amplification indicating the presence of *Ds* at the original location in TNP-29. b) *A*mplification indicating the presence of *Ds* transposon in the TNP-29 genome. c) Original flanking sequence amplification indicating the presence of *Ds* at the original location in TNP-79. d) Amplification confirms the presence of *Ds* transposon in the genome of TNP-79. "M" indicates a molecular weight marker. H_2O and GP are negative controls; original TNP-29 and TNP-79 lines are used as positive controls. Arrows indicate the expected band size of each PCR amplification.

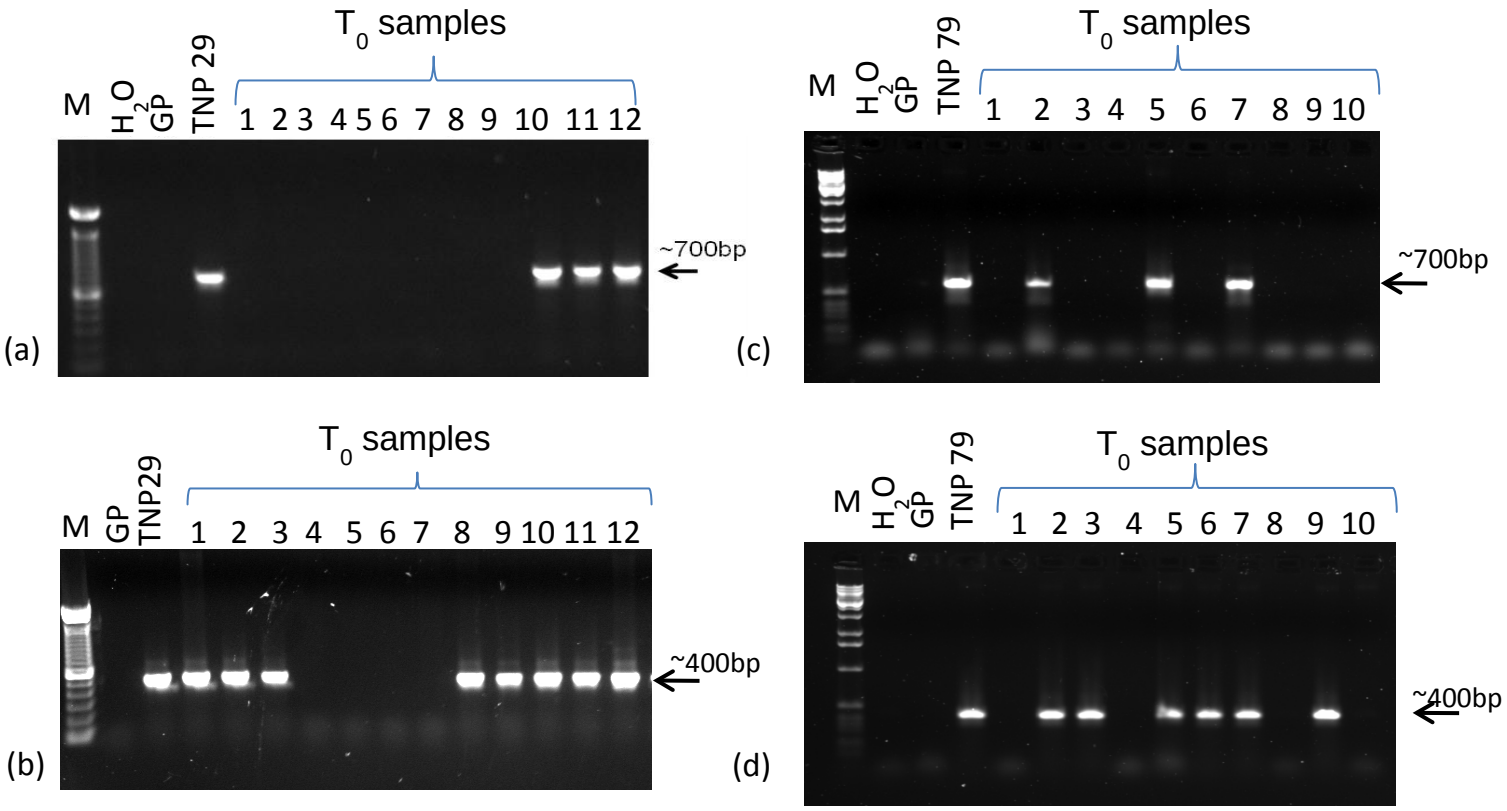


Figure 3.5 Diagrammatic illustration of flanking sequences showing the positions of exon, intron, the *Ds* insertion site, genomic regions, the putative start and stop codon and domain positions.

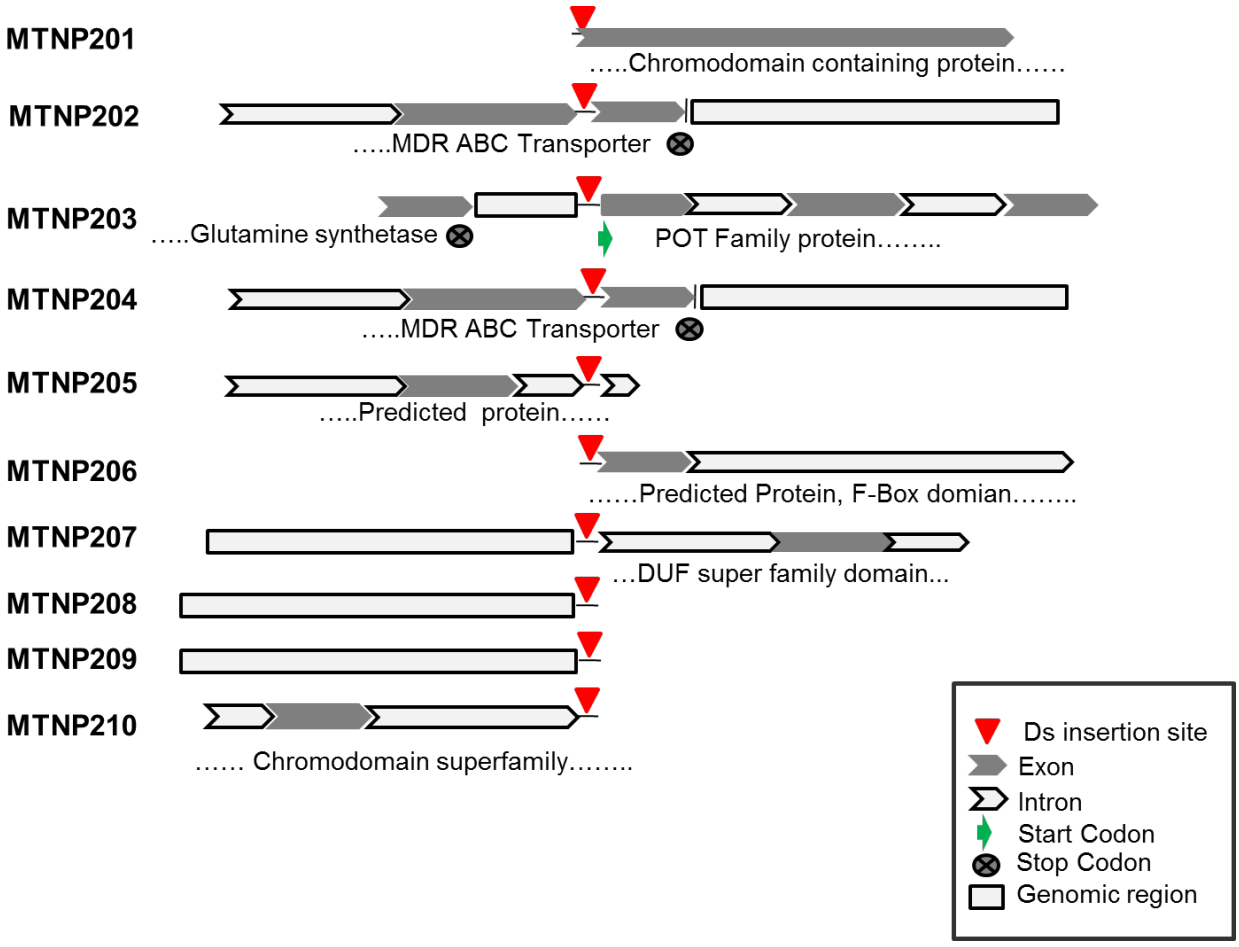


Table 3.1: Primer pairs used in amplification of *Ds* sequence, flanking region of *Ds* sequence, *Ac*TPase, empty donor sequence and primers used in iPCR protocol.

Primer pairs	Orientation	Sequence (5'→ 3')	Details
JNOSF	F	GCGCGGTGTCATCTATGTTACTAGATC	*Ds* Specific
JDS3R	R	TATCCCGATCGATTTCGAAC	
AC3	F	ACCACCAGCACTGAACGCAGACTC	*Ac* Specific
AC5	R	AACCTATTTGATGTTGAGGGATGC	
JIPF1	F	AACTAGCTCTACC GTTTCCG	Flanking (TNP-29)
JPT293R	R	TACGAACGCACAAGTCACAC	
JIPF2	F	CATATTGCAGTCATC CCGAA	Flanking (TNP-79)
JPT793R	R	CTTCAGAGCAGTCGCATAGT	
JPT295R	F	GGTCG ACACCTCCACTG TAG	Empty Donor (TNP29)
JPT293R	R	TACGAACGCACAAGTCACAC	
JPT795R	F	GGAGCCATGAGTAGGATTGT	Empty Donor (TNP79)
JPT793R	R	CTTCAGAGCA GTCGCAT AGT	
JIPF4	F	CTCGTGTTGTTCTGAGCGCACACA	iPCR
JIPR3	R	CGACCGGATCGTATCGGT	
JIPF2	F	CATATTGCAGTCATC CCGAA	iPCR
JIPR9	R	TGCGGAACGGCTAGAGCCAT	
JIPF7	F	CTAGATCGGCGTTCCGGT	iPCR
JIPR5	R	TGCGGAACGGCTAG AGCCAT	
JIPF1	F	AACTAGCTCTACC GTTTCCG	iPCR
JIPR6	R	TAGCAGCACGGATCTAACAC	

Table 3.2: Summary of *Ds* transposition in conventional and tissue culture approaches.

	Conventional approach		**Tissue Culture**	
	TNP-29X	**TNP-79X**	**TNP-29**	**TNP-79**
Total number of plants screened	370	223	46	64
Total New *Ds* Transposition	39	22	16	25
Stable New *Ds* Transposition	17	13	11	22
Unstable New *Ds* (with Transposase)	22	09	05	03
Reactivation Frequency	10.54%	9.80%	34.7%	39.06%

Table 3.3: Analysis of *Ds* flanking sequences and status of terminal inverted repeats (TIRs).

Line	Sequence side	Method	TIRs	Similarity	e-value	8bp Duplicates	GO Number
TNP- 29	3' and 5'	(iPCR)	Perfect	L-iditol 2-dehydrogenase	1.10E-06	TCCCCAGC	-
TNP-79	3' and 5'	(iPCR)	Perfect	Wheat EST BF200383.1	2.70E-06	CCCAGGGA	-
MTNP-301	3'	(iPCR)	Defective	Chromodomain protein	3.00E-20	CTTAACCT	GO:0006325
MTNP-302	3' and 5'	(iPCR)	Perfect	MDR-line *ABC transporter*	1.00E-18	CAATGGTG	GO:0042626
MTNP-303	3' and 5'	(HE-TAIL PCR)	Perfect	POT family protein	5.00E-39	GTTTCAAA	GO:0006542
MTNP-304	3' and 5'	(iPCR)	Defective	*β Amylase* like gene	2.90E-61	GTCGTGGC	GO:0042626
MTNP-305	3' and 5'	(HE-TAIL PCR)	Perfect	Barley BAC 673I14	6.00E-61	CCCCCACC	-
MTNP-306	3'	(HE-TAIL PCR)	Perfect	*Hordeum vulgare β GAL1* gene	2.00E-31	TGTGCAAA	-
MTNP-307	3' and 5'	(HE-TAIL PCR)	Perfect	*H. Vulgare* FLbaf82i04 mRNA	4.00E-24	CATAGAGT	GO:0005886
MTNP-308	3'	(HE-TAIL PCR)	Defective	*Zea mays* clone Contig887.FmRNA	3.00E-51	GTCGAGCT	-
MTNP-309	3'	(HE-TAIL PCR)	Perfect	No significant similarity	-	GTCCCGAG	-
MTNP-310	3'	(iPCR)	Perfect	*Hordeum vulgare* cDNA clone, mRNA	5.00E-24	TGCATGTA	GO:0006325

3.5 Discussion

Transposon based gene tagging methodologies were established initially in plant species which were easy to transform and for which complete genome sequences were available, such as *Arabidopsis thaliana* (Kuromori et al., 2004; Marsch-Martínez and Pereira, 2011) and rice (Izawa et al., 1997; Kolesnik et al., 2004; Qu et al., 2009). In larger genome species, like barley and wheat, the development of transposon-based functional genomic resources have been delayed, because their widespread synteny with rice was overemphasized. It is now known that extensive variation exists between these genomes (Bennetzen et al., 2005; Caldwell et al., 2004). For example, comparison of the rice genome with that of wheat, barley, maize and sorghum reveals that rapid evolution resulted in significant DNA sequence variation (Keller and Feuillet, 2000) among monocotyledonous species. Barley is a true diploid and shares a high degree of synteny with wheat; hence, developing an effective tagging system in barley will be useful for both these species. Also, barley is a significant industrial crop due to its use in the brewing and distilling industry. Malting quality is a complex trait which hinders barley's genetic improvement. Genetic mapping studies, using 9 different mapping populations, have identified more than 250 QTLs associated with 19 malting traits (Zale et al., 2000; Wei et al., 2009). Among these mapping populations, the malting quality QTLs were coincident (Zale et al., 2000). Limited studies have been conducted to identify genes associated with these QTLs. One major QTL complex, QTL2 mapped on the short arm of chromosome 4H, shows significant effects on malting quality parameters thus warranting its detailed characterization. The present study has developed new transposon insertion lines by reactivation of *Ds* from a malting variety, Golden Promise (Sato et al., 2011) mapped in the vicinity of malting quality regions on chromosome 4H. The characterization of new *Ds* mutants associated with the malting-related genes identified through other means will provide valuable insight into the genetics and physiological function of these genes.

Previously, genome-wide gene expression studies have identified candidate genes affecting malting. Expression studies using qPCR (Potokina et al., 2006; Vinje et al., 2011), microarray (Close et al., 2004; Potokina et al., 2004; Watson and Henry 2005)

and LongSAGE (White et al., 2008) have shown the likely role of many malting genes such as α and *β amylase, Cxp1.* at different stages of seed germination. Although these studies provide information about the stage and level of malting gene expression, new mutants are required to characterize these genes and the development of such mutants is one of the main objectives of current studies. *Ac*/*Ds* transposon-based mutants are a versatile system for gene discovery especially when sequence information is not available. In the present study, using conventional and *in vitro* approaches, a new source population with 108 new transposants was created by *Ds* reactivation from TNP-29 and TNP-79 to facilitate saturation of the 4H malting-quality region with *Ds* insertions. A single copy *Ds* from these two lines was genetically mapped on chromosome 4H near the malting-quality QTLs (Cooper et al., 2004).

To ensure the maximum utilization of the *Ac/Ds* system in barley, the approach to generating these lines was also refined. In the conventional genetic approach, the frequency of new *Ds* transpositions was found to be 10.5% and 9.8% for TNP-29X and TNP-79X populations, respectively. These results concur with previous publications regarding *Ds* reinsertion frequencies, which ranged from 11.8% to 17.1% in barley (Singh et al. 2006). In a comparative analysis of *Ds* transposition, the same TNP lines used in the conventional approach were subjected to a tissue culture approach. A significant increase in *Ds* transposition frequency was achieved through *in vitro* techniques compared with the conventional genetic technique. A reactivation frequency of 34-39% was observed, far greater than the *Ds* transpositions generated through conventional methods (9-11%). It has been speculated that developmental reprogramming during tissue culture leads to alteration in chromatin structures and erases the 'silencing environment' activating the transposable elements (Chul et al., 2002). Genetic and molecular experiments in maize led to the hypothesis that the DNA methylation status of *Ac/Ds* elements determines their transpositional competence. Methylation of *Ds* termini is the most likely reason for the silencing of *Ac*. Tissue culture resets methylation and it has been shown that *Ac* transposase can bind to unmethylated *Ds* ends (Ros and Kunze 2001; Smulders and Klerk 2011). The higher activity of *Ds* transposition observed in our tissue culture approach may be due the above-mentioned facts. In addition, variation between two launching pads (TNP-29 and -79) was

observed; higher *Ds* reactivation frequency was observed in TNP-79 than in TNP-29 (39% vs. 30%). This may be due to the position of *Ds* in the launching pad. A similar position effect has been observed in relation to the *Mu* element in corn (Singh et al., 2008).

Our studies demonstrated reactivation of *Ds* through the transient expression of *Ac*TPase. This is an important observation as *Ac*TPase is prone to silence after a few generations thereby hindering the movement of *Ds* elements. Reduced activity or complete silencing of the *Ac*TPase transposase had been previously detected in barley (Koprek et al., 2000) and other plant species (Chin et al., 1999; Greco et al., 2003; Kohli et al., 2004). For example, genome-wide silencing of *Ac*/*Ds* transposons was observed in maize (Brettell and Dennis, 1991). In heterologous species, the decline in *Ds* transposition frequencies has also been correlated with reduced expression of *Ac*TPase. Studies in rice have confirmed a loss or reduction of *Ds* mobility during generation advance due to a pronounced reduction of *Ac*TPase expression by epigenetic silencing (Nakagawa et al., 2000; Upadhyaya et al., 2002). Hence, transient expression of *Ac*TPase and reactivation of *Ds in vitro* can efficiently saturate important regions and QTLs with *Ds* to facilitate the cloning and dissection of such regions.

Ds flanking sequences from a subset of new lines were analyzed using BLAST. The new flanking regions showed similarities to known proteins, genomic sequences and/or ESTs. These results are consistent with previous findings in maize (Cowperthwaite et al., 2002), rice (Kolesnik et al., 2004) and barley (Singh et al., 2006). New *Ds* insertions have been found in exons or close to exons, a finding which is crucial for functional genomics studies in the large and repetitive genome of barley. Based on the analysis, most of the *Ds* flanking sequences (Table 3.3; Figure 3.5) are predicted to affect malting quality traits and are shown to be expressed in various stages of seed germination and in tissues such as scutellum, embryo and endosperm. In this study, most notable *Ds* tagged genes include the *β-amylase gene* and *β-GAL1,* which play important roles in malting and seed germination (Triantafillidou and Georgatsos, 2001). Our EST analyses indicate that several genes tagged by *Ds* were differentially expressed in relation to malting. However, no mutants are available for these important genes, thus hindering

their further characterization. Therefore, newly developed *Ds* mutants would be a valuable resource for investigating their role in malting.

Various other putative genes tagged by *Ds* include chromodomain-containing protein, an ABC transporter protein, an F-Box protein, a POT family protein, and a glutamine synthetase. The development of knock-out lines in these genes and in other tagged, ESTs of unknown function provides useful starting material for functional studies. Our ongoing mapping effort of these flanking sequences will facilitate further studies by providing a link between malting quality QTLs on chromosome 4H and these putative genes.

3.6 Acknowledgements

This work was supported by the Brewing and Malting Barley Research Institute and the Natural Sciences and Engineering Research Council of Canada Collaborative Research and Development. We also thank Dr. Ravneet Kaur and Dr. Manjit Singh for critically reading this manuscript. The authors also thank Mr. Neil Dylan Lamb-Palmer for his suggestions about bioinformatic analysis.

CONNECTING STATEMENT BETWEEN CHAPTER III & IV

New *Ds* insertion lines were generated in the previous study (Chapter III), to saturate the malting quality QTL located on chromosome 4H. The insertion site needs to be located on the barley map. As described in chapter III, the *Ds* transposon was reactivated through conventional breeding methods by crossing single *Ds* insertion lines TNP-29 and TNP-79 with an *Ac*TPase expressing line 25-B, and using tissue culture methods, by extra-chromosomal expression of *Ac*TPase. In the following study, we generated additional flanking sequences and mapped all of the *Ds* insertions (Singh et al., 2012 and the current study) on the barley linkage map. They were mapped using a public database of whole genome sequence of barley and some were validated by sequence based polymorphism using different mapping populations. Integration of various mapping resources with the *Ac/Ds* transposon system will allow localization of *Ds* knockout genes on chromosome for further saturation mutagenesis and tagging of linked genes by *Ds* remobilization.

I contributed to all the work described in chapter IV, including designing the experimental set-up, conducting all the experiments and writing the manuscript. Experiments include: Generation of new *Ds* insertions, Polymorphism detection, bioinformatic analysis and mapping studies. Some of these results were included in manuscript recently accepted in “Molecular Breeding”. Dr. Jaswinder Singh provided funding for this project through an NSERC-CRD grant.

Chapter IV

Generation of *Ds* flanking sequences and their location on barley genetic linkage map

Surinder Singh, Nandha P and Jaswinder Singh*

*Corresponding Author: Dr. Jaswinder Singh

Plant Science Department, 21 111 Rue lakeshore

McGill University, Quebec, H9X 3V9, Canada

Email: jaswinder.singh@mcgill.ca

Phone: +1(514) 398-7906

Singh S, Nandha P, Singh J (2015) Insertion site structure of mapped barley *Ds* loci in QTL2 region (Manuscript in preparation for **Genome**).

4.1 Abstract

Among various functional genomics tools used to characterize genes in plants, transposon-based mutagenesis approaches offer great potential, especially in barley and wheat, which possess large genomes, and in which genetic transformation is not routinely possible. A *Ds* transposon insertion line (TNP), TNP-29 was previously mapped in the vicinity of a malting quality QTL, known as QTL2 located on chromosome 4H of barley. Reactivation of a *Ds* transposon from this TNP line led to the identification of genes in this QTL2 region. We generated several *Ds* (dissociation) lines by crossing TNP-29 with *AcTPase* (activator) expressing line (25B), and F_2 progeny were screened for *Ds* insertions at new locations. Further characterization of these *Ds* mutants, requires location of their position on the barley genetic map. In the present study, we mapped the new *Ds* insertions and found that 19 % were in the QTL2 region. Mapping was carried out using a sequence based approach, and a linkage map was generated updating the current map. We also confirmed the map position using data from the barley sequence database (IBGSC 2012). Locating these *Ds* loci on the barley map will lead to understaning of the QTL2 malting region for facilitating identification of candidate malting genes.

4.2 Introduction

Barley has large genome size of approximately 5.1Gb (IBGSC 2012), and more than 80% of the genome consists of repetitive DNA sequences (Sreenivasulu et al., 2008) making gene cloning studies cumbersome. Based on collaborative international efforts, impressive progress has been made in establishing freely available, public resources in barley genomics – an advantage not realized in a number of other major crop species (Gill et al., 2004). These resources include the development of extensive genetic maps. Currently, most linkage maps developed in barley are curated and are available on GrainGenes (http://wheat.pw.usda.gov/GG2/maps. shtml#barley; verified, Dec. 2014). Recently a high-density genetic linkage map based on two enzyme genotyping by sequencing (Poland et al., 2012) and Restriction Site Associated DNA (RAD) (Chutimanitsakun et al., 2011) were developed in barley. These molecular marker linkage maps have been useful for identifying QTLs (Hayes et al., 2003) and are essential for mapping of ESTs and other unknown genes, most of which still have no assigned function.

Integration of these mapping resources with the *Ac/Ds* transposon system allows localization of *Ds* knockout genes (Singh et al., 2012) on chromosomes for saturation mutagenesis and tagging of linked genes by *Ds* remobilization. Mapping of *Ds* loci can be achieved through identification of polymorphisms in the sequences flanking *Ds* that are generated through iPCR, TAIL PCR and Adapter ligation techniques. A set of *Ds* insertions described in Singh et al. (2012) has been constructed in an old malting variety Golden Promise, in which successful transformation of barley was first accomplished (Wan and Lemaux 1994). However, Golden Promise is not a parent of the well-characterized mapping population currently available (http://wheat.pw.usda .gov/ggpages/map_summary.html). Thus, we utilized other highly polymorphic mapping populations; like Oregon Wolfe Barley dominant (OWB-D) X Recessive (OWB-R), Steptoe X Morex and Dicktoo X Morex. Among all mapping techniques, sequence based approach was proven successful as used earlier to map 19 *Ds* loci on barley linkage map (Cooper et al., 2004). SNPs and insertion deletions (INDELs) are the most common type of sequence difference between alleles (Rafalski 2002) and have been

used in mapping ESTs in barley. Restriction digest-based assay of PCR products, generally known as CAPS (Cleaved Amplified Polymorphic Sequence) is also a preferred method for performing SNP assays in mapping populations. Further, more recently created barley sequence database (IPK: http://webblast.ipk-gatersleben.de/barley/) provides insight into the physical and genetic map location of *Ds* loci (IBGSC 2012). The overall aim of this study was to identify *Ds* loci mapping in the malting QTLs for future identification of genes involved in malting quality. We mapped all the new *Ds* insertions generated previously (Singh et al., 2012) and the present study, to barley linkage map. Joinmap4 software was used to map the *Ds* insertions, and the position was confirmed using the barley sequence database.

4.3 Material and Methods

4.3.1 Generating *Ds* flanking sequences

Sequences of *Ds* flanking regions from new transpositions were obtained through HE-TAIL PCR (Tan and Singh 2011) and iPCR (Singh et al., 2012) and adapter ligation method (Brown et al., 2012). The HE-TAIL PCR was performed in four separate runs using genomic DNA, Takara ExTaq DNA polymerase, 10X PCR buffer containing 10 mM $MgCl_2$, and 2.5 mM dNTPs (Takara-Bio, Dalian, China). These PCR runs include pre-amplification, primary amplification, secondary amplification and tertiary amplification respectively, following the conditions described by Tan and Singh (2011), using GeneAmp® PCR System 9700 (Applied Biosystems, USA).

For iPCR, genomic DNA was digested with either *Nco*I or *Nhe*I (both enzymes have a restriction site in the *Ds* element), followed by heat inactivation and phenol:chloroform purification of the digested DNA. The digested DNA was self ligated and the product used for subsequent PCR. The cocktail for the first cycle of the iPCR is prepared by adding 1.0 µl of 0.1 µg of purified and ligated DNA, 2.5 µl of 10X buffer, 2.5 µl of 2.5 mM dNTP mixture, 0.5 µl of 10 µM of the first set of nested primers for the 5' end JIPR3and JIPF4 and the 3'end JIPR9 and JIPF2 and 0.25 µl of 1U of Ex Taq DNA polymerase (Invitrogen, USA) in a total reaction volume of 25 µl. PCR products from the first round were then diluted to 1:20 in the 5' end and 1:50 in the 3' end with water and used as

template for the second round of PCR. Specific PCR products were generated in the second round of PCR by amplifying with nested primers (JIPR5 and JIPF7 on 5´ side and JIPR6 and JIPF1 on 3´ end). The products obtained from second iPCR were gel purified using gel extraction kit (QIAquick® Gel extraction kit, Qiagen, USA).

In the adapter ligation (ALPCR) method, adapters with appropriate overhangs were designed that include a specific primer binding site (A*C946R*), used with four set of restriction enzymes (AseI, HinfI, AvaII, HhaI). These enzymes were selected based on the position of restriction sites in the modified sequence (*Ds*-bar, consisting of minimal 5' and 3' *Ds* termini flanking a ubiquitin-driven bar cassette). Adapters were ligated to the restricted genomic DNA, and three rounds of PCR with three sequentially nested primers specific to 3' and 5' termini of known sequences were carried out.

4.3.2 Bioinformatic analysis of the new flanking sequences

Bioinformatic analysis of the flanking sequences was carried out using a BLAST algorithm (Basic Local Alignment Search Tool). EST's and proteins expression related to the flanking sequences were queried against the EST database HarvEST (http://harvest.ucr.edu), NCBI (http://www. ncbi. nlm.nih .gov/) and Gramene (http://www.gramene.org). Flanking sequence and EST alignment were performed with clone manager 9 software. GenScan (http://genes.mit.edu/) was used to predict coding sequences. For describing gene product characteristics and gene product annotation, GO (Gene Ontology) analysis was also carried out (http://www.geneontology.org/).
The flanking sequence information was also the basis for determining orientation of *Ds* insertions and structure of insertion sites, including terminal inverted repeats (TIRs) and 8 bp duplications, and to characterize the genomic regions in which transposition occurred.

4.3.3 Localization of *Ds* flanking sequences on barley chromosomes

4.3.3.1 Mapping population

Seeds for the highly polymorphic mapping population, Oregon wolf barley (OWB) OWB-D X OWB-R (94 F1 individuals) were grown in green house, and DNA was extracted. Methods for DNA sample collection and extraction was as in Singh et al. (2012). Other mapping populations such as Steptoe X Morex and Dicktoo X Morex were also used in case no polymorphism was detected in OWB population.

4.3.3.2 Primer design and polymorphism detection

Ds flanking sequences developed in Singh et al. (2012) and the current study were used for mapping *Ds* loci. Primers specific to 21 *Ds* flanking sequences are designed using “clonemanager9” software (Table 4.1). These primers were used to amplify parents of various mapping population i.e., OWB-D, OWB-R, Steptoe, Morex, Dicktoo and Golden Promise (Control) varieties. PCR amplification products were gel purified and sequenced at the Genome Quebec sequencing center, McGill University. Polymorphism (SNPs/INDELs) was detected in the amplification products by comparing mapping parents and Golden Promise (GP), using freely available software BioEdit (http://bioedit.software.informer.com/) (Hall 1999).

To detect INDELs, PCR products were analyzed on high concentration agarose gel (3-4%). Presence or absence (dominant) and size difference (co-dominant) in amplification bands were scored using mapping software. In the case of SNPs, sequences were analyzed for any alterations in restriction sites in respect to change in the nucleotide. This allowed us to generate CAPS for easy scoring of polymorphism. For the CAPS assay, PCR amplicons were digested with specific restriction enzymes before separating by agarose gel electrophoresis.

4.3.4 Barley sequencing database

IBGSC has published a barley genome with access to sequence and annotation data as well as a physical/genetic map. IPK barley (http://webblast.ipk-gatersleben.de/barley/) provides a database for BLAST searches against a whole genome sequence assembly, BAC, Full-length cDNA, ESTs and exome of barley. The barley database was employed to infer map location of *Ds* loci, for which no polymorphism was detected using sequence-based methods.

4.3.5 Linkage map construction and statistical analysis

A linkage map of a chromosome is constructed by determining how frequently two markers are inherted together. “JoinMap 4” was used to develop a linkage map for *Ds* loci. An updated linkage map was constructed using the most recent version of the OWB map (Chutimanitsakun et al. 2011), available on grain genes. Raw data were downloaded from http://wheat.pw.usda.gov/ggpages/maps/OWB/

Barley contains seven linkage groups, which correspond to seven barley chromosomes. Segregation data of SNPs/INDELs obtained from polymorphism data were used for linkage analysis and sequentially added to linkage group. Allele data for 94 doubled haploid (DH) lines from the OWB mapping population constituted linkage map construction. On the basis of recombination frequency, marker order was assigned to the linkage map, and subsequently map distance (in cM) between markers was calculated. Linkage groups were identified using minimum LOD values of 5. The Monte Carlo Maximum Likelihood (ML) mapping algorithm was used to determine the orders of markers within each linkage group. Map distances were calculated using Haldane’s mapping function. Maps were drawn using MapChart v2.2 (Voorrpis 2002).

4.4 RESULTS

4.4.1 Characterization of flanking sequences

Flanking sequences generated using AL-PCR (adapter ligation), HE-TAIL PCR, and iPCR methods were analyzed using various bioinformatic tools. Based on a BLAST search, the majority of TNP flanking sequences matchd known ESTs or a characterized gene sequence. Searches in the HarvEST database showed that the MTNP-315, -317, -320 and -321 lines possess high homology to ESTs expressed in malting stages. They are candidates for further study of different malting traits. Table 4.2 presents the information about the flanking sequences their gene homology, pedigree, state of terminal inverted repeats (TIRs) and methods used to generate them. The majority of *Ds* insertions are within or near to a genic region. Table 4.2 also mentions 8bp sequence duplicates (characteristic of *Ds* transposition) which correlate with the perfect or defective TIRs. *Ds* insertions with defective TIRs will be unavailable to transpose to new locations in the next generation.

4.4.2 Structure of *Ds* insertion sites

To determine the nature of *Ds* insertion sites in the barley genome, analyses of *Ds* flanking sequences were conducted using BLAST searches of public databases, such as those at the harvEST, Gramene and NCBI websites, looking for similarities to known proteins, genomic sequences, and/or ESTs. Based on bioinformatic analysis we found the majority of the *Ds* flanking sequences matches with known ESTs or characterized gene sequences. Analysis of these flanking sequences with gene prediction programs and bioinformatic tools indicated their homology to various genes and proteins. Figure 4.1 depicts the positions of exons, introns, the *Ds* insertion site, genomic regions, the putative start and stop codon and domain positions of MTNP lines generated in this study.

4.4.3 Chromosome localization of *Ds* flanking sequences on the barley map

4.4.3.1 Investigation of polymorphisms

It is highly desirable that *Ds* flanking insertions sequences should be polymorphic among mapping population parents, prior to testing their amplification in all individuals of a mapping population. Specific primers generated from the flanking sequences (Table 4.1) were used to amplify the parent of mapping population individuals (OWB D, OWB R, Steptoe, Morex, Dicktoo). We observed SNP or INDEL polymorphism among the parents of mapping population. SNPs were converted to a cleaved amplified polymorphic sequence (CAPS) assay with a restriction enzyme that cut one parent but not the other. Figure 4.2 shows polymorphisms detected in two of the *Ds* insertion lines, in different mapping populations. In MTNP 301, restriction enzyme cuts the PCR product of OWB R but not OWB D, and in MTNP 310 excision occured in Morex and not in Steptoe mapping population parents. All individuals (here shown 15 from both the population) were restricted with the same enzyme and data recorded in the form of similarity to parent A or parent B. For sample sequences in which no polymorphism was detected, we employed whole genome sequencing barley database to infer the position of *Ds* loci.

4.4.3.2 Map location of *Ds* insertions

Ds flanking sequences generated in Singh et al., (2012) and in the current study were mapped on the barley linkage map. The most recent barley map of the OWB mapping population available on GrainGenes was used to locate *Ds* insertional sequences (http://wheat.pw.usda.gov/ggpages/map_shortlist.html). We used genome sequencing resources available on barley sequence database (http://mips.helmholtz-muenchen.de/plant/barley/) (IBGSC 2012) to determine the location of *Ds* insertions. The database provides access to physical/genetic map and barley Blast server to locate the position of new *Ds* transposons lines. Each *Ds* loci was added to the barley genetic map, and an individual map was generated for each chromosome. *Ds* insertions were located on all barley chromosomes except 6H. Figure 4.3 depicts inferred location of the *Ds* insertions (MTNP lines) on different barley chromosomes (1H to 7H), using the

barley sequence database. Results are summarized in Table 4.3, with chromosome location and distance of each locus in centimorgans (cM). A linkage map based on the OWBD X OWBR and Steptoe X Morex mapping populations was generated using JoinMap4 software to confirm the position of two of the MTNP lines (Figure 4.4).

4.4.4 Frequency of linked *Ds* transposition

We generated 41 new *Ds* insertions by reactivating candidate TNP lines; TNP-29 and TNP-79. Thirty-three lines were generated from TNP-29A (TNP- 29 X *Ac*TPase), and eight lines from TNP-79A (TNP-79 X *Ac*TPase). Using HE-TAIL PCR, iPCR and AL methods, we generated 22 flanking sequences. All were mapped using the barley WGS database and confirmed with linkage mapping. Five mapped sequences were from TNP-79 and 17 from TNP-29. Two of five TNP-79 loci were mapped on same the chromosome (4H); one near the launch pad site and other about 40 cM away in a telomeric region. Similarly three of 17 TNP-29 loci were mapped in the QTL2 region about 20-45 cM from the telomeric region. TNP-79 depicted 40% frequency into linked positions and TNP-29 showed 19% linkage frequency. Altogether, five *Ds* loci were mapped on chromosome 4H, with four in the QTL2 region (Figure 4.5).

Table 4.1: Primers used to amplify parents of various mapping populations to detect polymorphism.

Line	Primer Orientation	Primer sequence (5'→ 3')	Line	Primer Orientation	Primer sequence (5'→ 3')
MTNP 301	F	TCTGCCGTCTGTTGTGGTTC	MTNP 311	F	ATGGCGGTCCTTATGTCTTG
	R	GCTTCTCGCTTGTGCCATTG		R	GCTGCCTGTCCATTCAATAG
MTNP 302	F	TGGGCATCAAGGGTGGAGTC	MTNP 312	F	TTGCCCTCCTTAGAGATGTG
	R	CATCGGCATTCGGCAGTAGC		R	GGCGATATACCTTTCTACCG
MTNP 303	F	CACCAGCGACGGATCTGTTG	MTNP 313	F	CAGGAGGATAGCTTGAAGTG
	R	ACGGTGGTATGGACCGGAAG		R	AGGGAGGAGGTGAATCAAAC
MTNP 304	F	GGTCGAGCTACACTCAAAGG	MTNP 314	F	GGTGCCTTAACGCTCGAATG
	R	CTTAGGGCAGCAATGTCAGG		R	GGGCCACTGAGAATTTCTGC
MTNP 305	F	GTGCTTCACCCGCTAATCTG	MTNP 315	F	GATGTTGCGGTTGCCATAAG
	R	GGAGTCCCGTCAATGCTTTC		R	CGAGAGGTACGACTACATTG
MTNP 306	F	ACCACCTTCTAATCGCAGAG	MTNP 316	F	GAAGCCACATGGCAGCCAAG
	R	TAGCACCAGCCGTTCAAATG		R	CATCCGCGCAGACAAGCAAG
MTNP 307	F	TAGTACAATTCGGGCTAGGG	MTNP 317	F	ACAATGGTGGTCCTGCTTAC
	R	CACGTACTTGGTGTGGTATG		R	CAATTACAGGCAAGCCATCC
MTNP 309	F	TTGTCCAGACGCCACACCAC	MTNP 318	F	GTGCTGGTTGGCAGGATGTC
	R	GTCGCCAGCAGCGTAACAAC		R	CACGTCTCGCCGATCTTCAC
MTNP 310	F	GGGAGCTTCTGATGTGTTTC	MTNP 320	F	ATATCCGCCAGGTGCAGCTC
	R	GCTGCCTGTCCATTCAATAG		R	TCTCGTCAGCGTGCAGTCAG
			MTNP 321	F	GTCATCATCCGGTCGTCCTC
				R	CTACCCACACGTCCCTTCTC

Table 4.2: Status of terminal inverted repeats, 8 bp duplicates and annotation of *Ds* flanking sequences.

Line	Sequence side	Method	TIRs	Similarity	e-value	8bp Duplicates
TNP-29	3' and 5'	(iPCR)	Perfect	L-iditol 2-dehydrogenase	1.10E-06	TCCCCAGC
TNP-79	3' and 5'	(iPCR)	Perfect	Wheat EST BF200383.1	2.70E-06	CCCAGGGA
MTNP-301	3'	(iPCR)	Defective	Chromodomain protein	3.00E-20	CTTAACCT
MTNP-302	3' and 5'	(iPCR)	Perfect	MDR-line *ABC transporter*	1.00E-18	CAATGGTG
MTNP-303	3' and 5'	(HE-TAIL PCR)	Perfect	Peptide transport PTR2	9.00E-49	GTTTCAAA
MTNP-304	3' and 5'	(iPCR)	Defective	*β Amylase* like gene	2.90E-61	GTCGTGGC
MTNP-305	3' and 5'	(HE-TAIL PCR)	Perfect	*Idg2* gene for isocitrate dehrogenase	4.00E-59	CCCCCACC
MTNP-306	3'	(HE-TAIL PCR)	Perfect	*Hordeum vulgare β GAL1* gene	2.00E-31	TGTGCAAA
MTNP-307	3' and 5'	(HE-TAIL PCR)	Perfect	*H. Vulgare* FLbaf82i04 mRNA	4.00E-24	CATAGAGT
MTNP-308	3'	(HE-TAIL PCR)	Defective	*Zea mays* clone Contig887.FmRNA	3.00E-51	GTCGAGCT
MTNP-309	3'	(HE-TAIL PCR)	Perfect	No significant similarity	-	GTCCCGAG
MTNP-310	3'	(iPCR)	Perfect	*Hordeum vulgare* cDNA clone, mRNA	5.00E-24	TGCATGTA
MTNP-311	5’	(HE-TAIL PCR)	Perfect	*Hordeum vulgare* serine/threonine kinase-like protein	4.00E-16	CTGGGGA
MTNP- 312	3’	(HE-TAIL PCR)	Defective	No significant similarity	-	TCGACTGA
MTNP-313	3’	(iPCR)	Defective	40S ribosomal protein-predicted	1.00E-82	TTCTAGTG
MTNP-314	3’	(ALPCR)	Perfect	Hypothetical protein TRIUR3 (*Triticum urartu*)	7.00E-176	CGAGCTGT
MTNP-315	3'	(ALPCR)	Perfect	*Hordeum vulgare* cDNA clone: FLbaf17m18	9.00E-120	GCCGTCGG
MTNP-316	3’	(ALPCR)	Perfect	No significant similarity found	-	TAAGAATC
MTNP-317	3’	(ALPCR)	-	*Brachypodium distachyon* ABC transporter	6.00E-108	Not Known
MTNP-318	3’	(ALPCR)	-	*Hordeum vulgare subsp. vulgare Lks2* gene	2.00E-08	Not known
MTNP-319	5’	(ALPCR)	Perfect	*Oryza sativa*, BAC clone: OSIGBa0103O01	2.00E-13	CCCAGTAC
MTNP-320	3’	(iPCR)	Defective	No Significant similarity	-	TGCCTCGC
MTNP-321	3’	(iPCR)	Defective	Predicted protein, complete cds, clone: NIASHv3026K04	2.00E-42	TATTTAAC
MTNP-322	3'	(ALPCR)	Perfect	DNA repair radA-like protein	5.00E-14	ATGGAAGG

Table 4.3: Origin of MTNP lines and their map location on barley linkage map.

TNP line	Origin	Chromosome location	Genetic linkage position (cM)
TNP-29	PDS2	4HS	27.4
TNP-79	PDS3	4HS	70.3
MTNP-301	TNP 29	7H	70.8
MTNP-302	TNP 29	3H	153.7
MTNP-303	TNP 79	4H	10.7
MTNP-304	TNP 29	3H	152.6
MTNP-305	TNP 29	4H	15.9
MTNP-306	TNP 29	1H	103.2
MTNP-307	TNP 29	2H	136.9
MTNP-308	TNP 29	7H	69.4
MTNP-309	TNP 29	2H	37.6
MTNP-310	TNP 29	7H	73.8
MTNP-311	TNP 29	4H	6.0
MTNP-312	TNP 29	1H	35.6
MTNP-313	TNP 29	5H	29.7
MTNP-314	TNP 29	5H	44.8
MTNP-315	TNP 29	4H	11.6
MTNP-316	TNP 29	5H	48
MTNP-317	TNP 79	4H	73.9
MTNP-318	TNP 29	3H	29.6
MTNP-319	TNP 29	2H	65.6
MTNP-321	TNP 29	3H	58.6
MTNP-322	TNP 29	5H	1.1

Figure 4.1: Diagrammatic representation of additional flanking sequences generated, showing the positions of exon, intron, the *Ds* insertion site, genomic regions, the putative start and stop codon and domain positions.

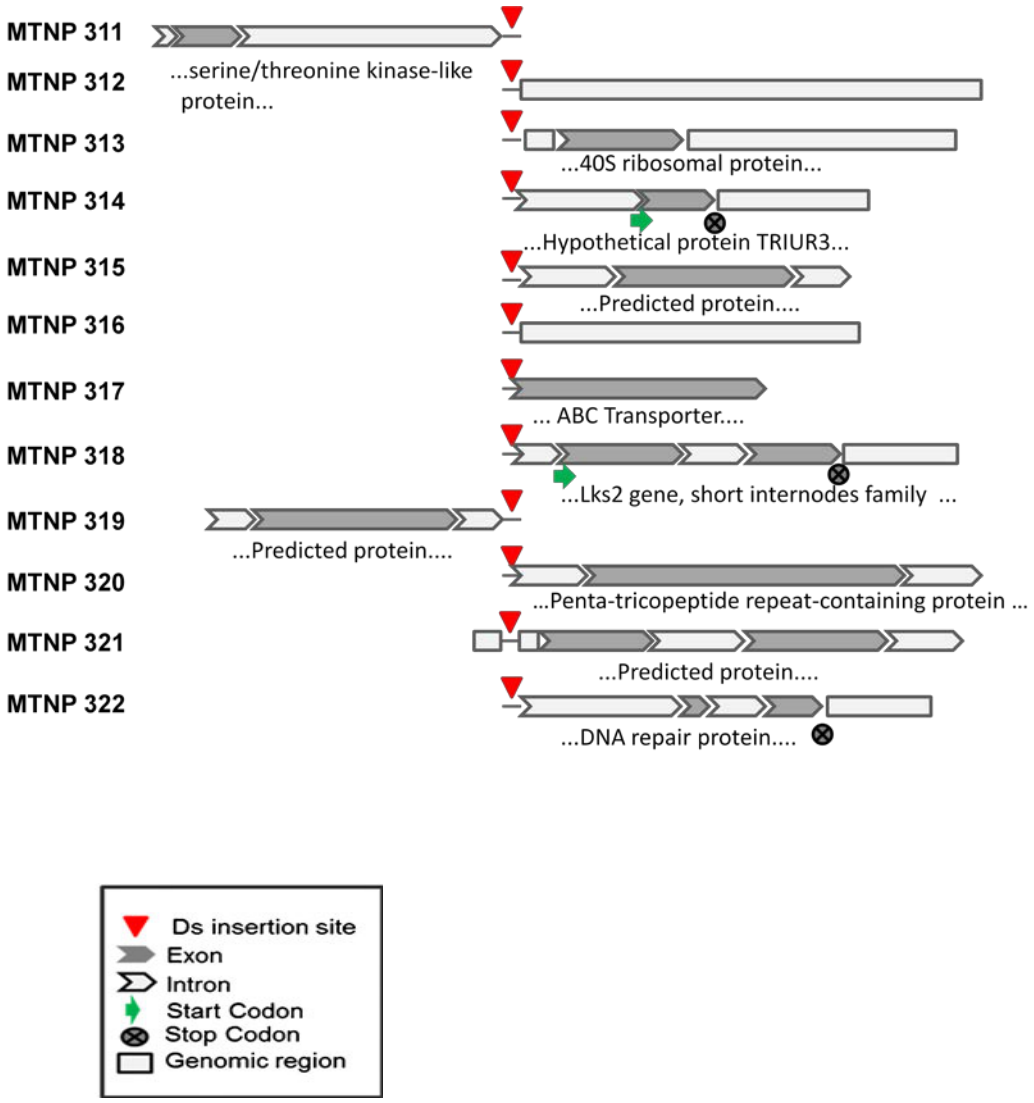
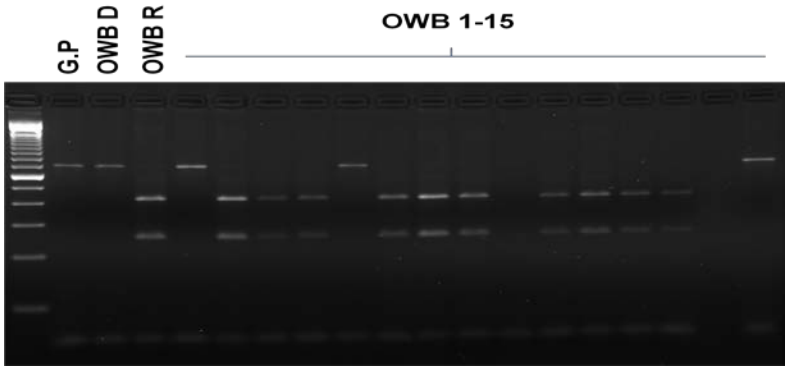


Figure 4.2: a) *Ds* Transposon insertion line MTNP 301, showing polymorphism as detected using a CAPs assay. A SNP detected in one mapping parent (OWB-R) has a restriction enzyme (sau1) site, which restricts all mapping population individuals with 'OWB-R type' and not the 'OWB-D type'. b) MTNP 310 showing a polymorphism detected in Steptoe X Morex population due to a SNP. Fifteen samples are shown here in each population as an example.

a)



b)

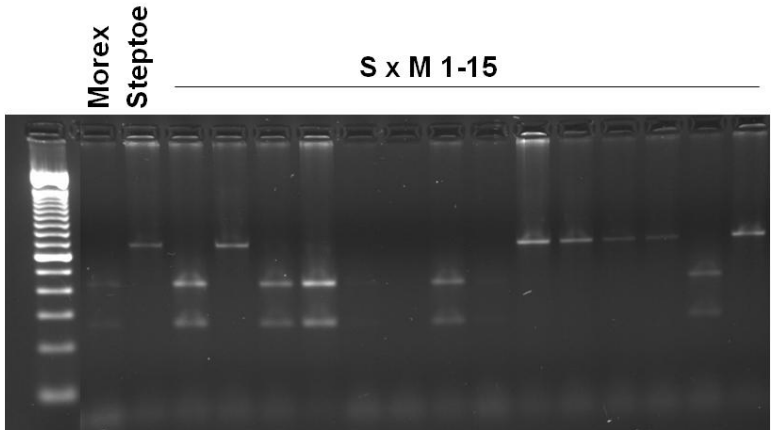
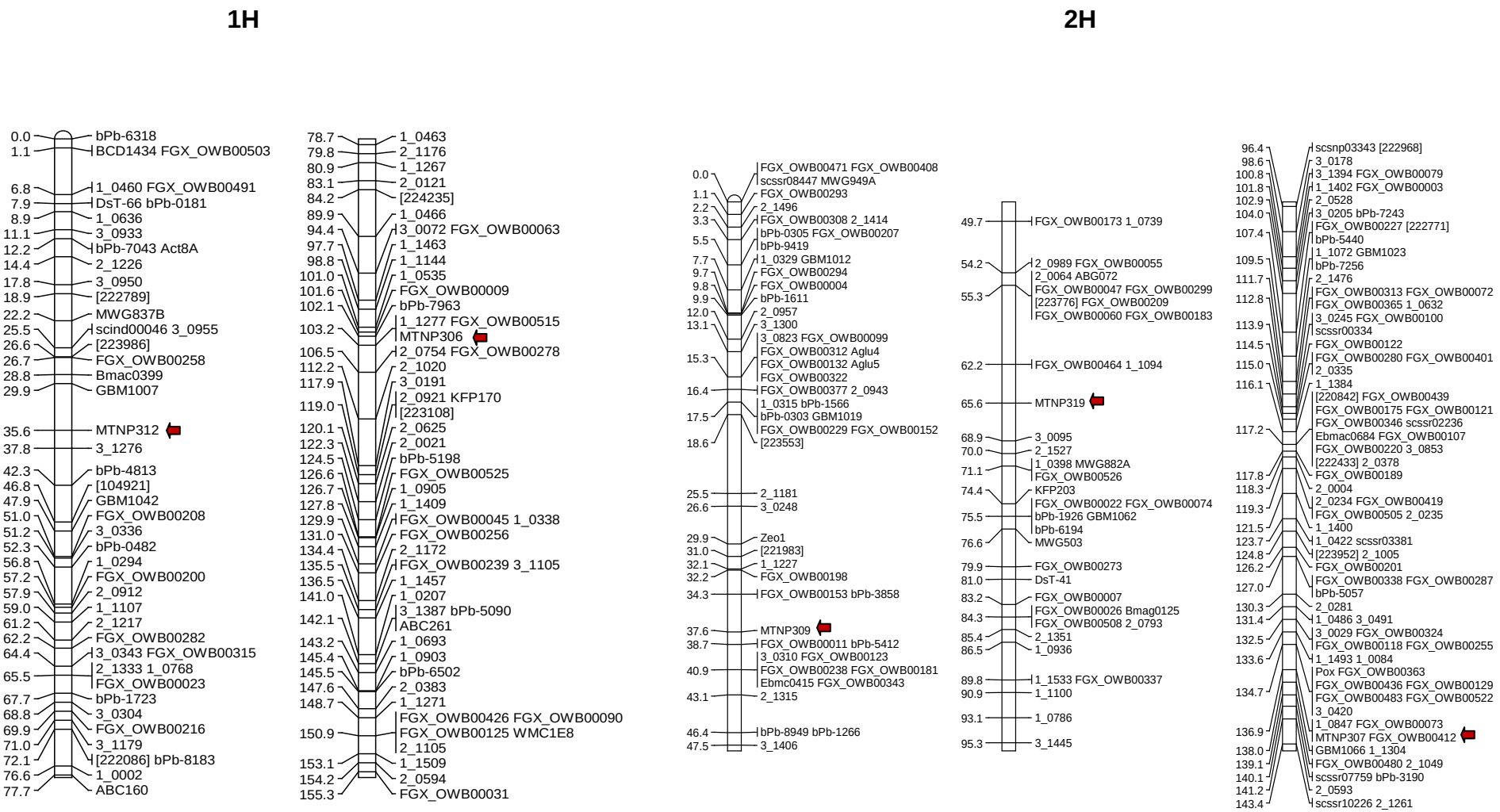
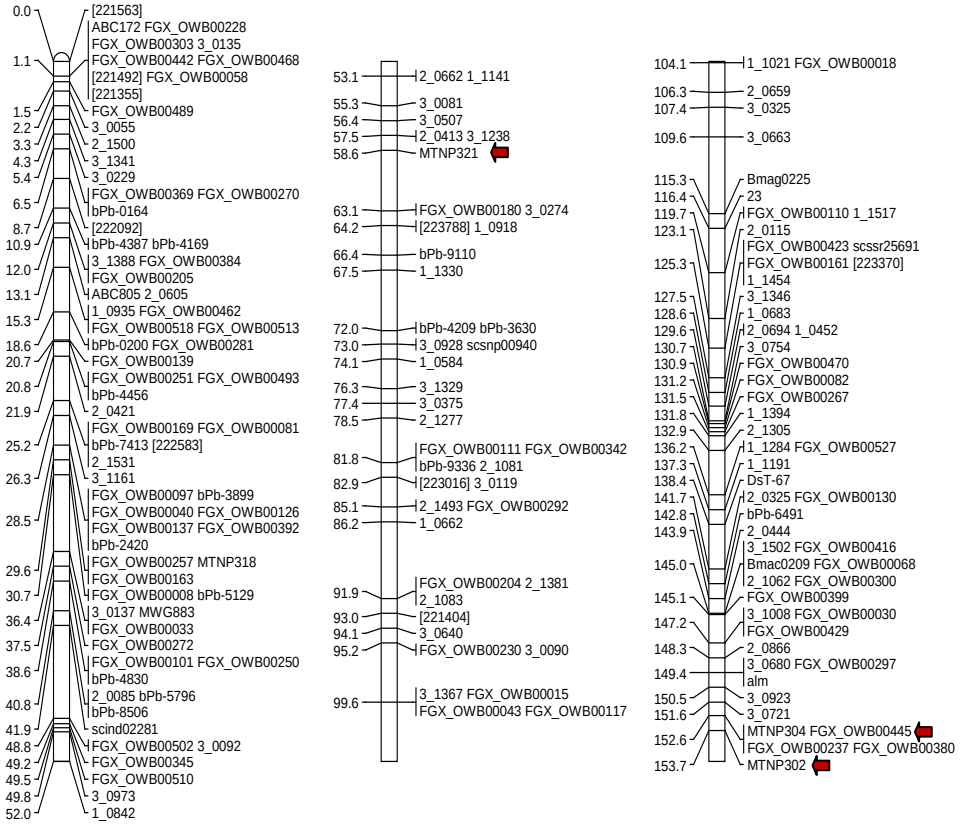


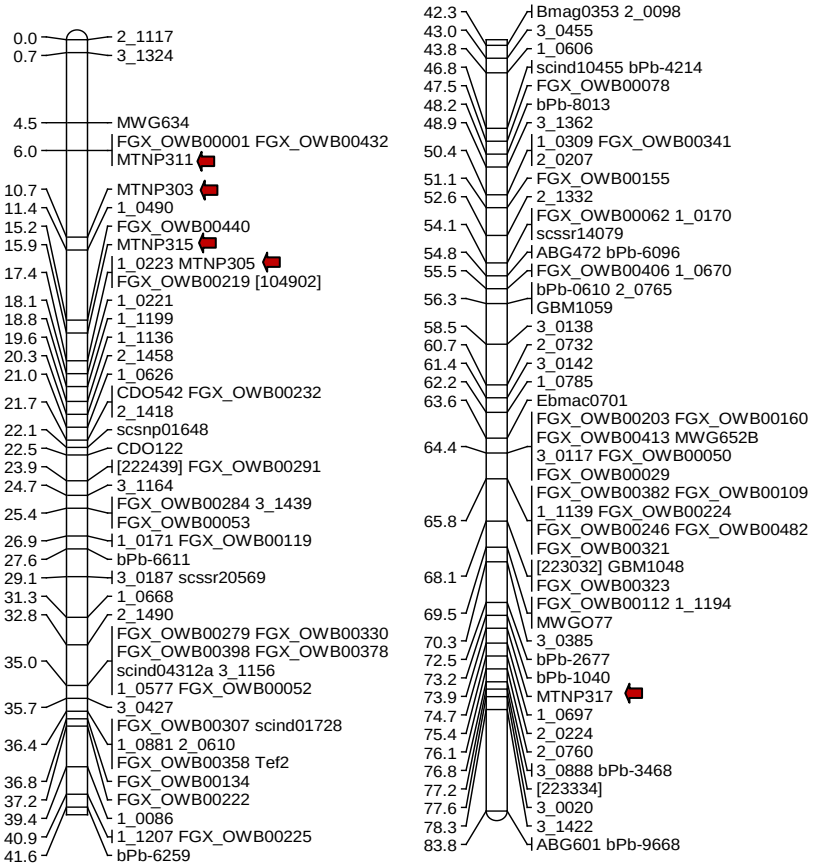
Figure 4.3: Barley linkage map showing MTNP lines located on Chromosome 1H to 7H as inferred from barley sequence database. Each MTNP line mapped on individual chromosome is mentioned below. Red arrows indicate the location of MTNP lines.



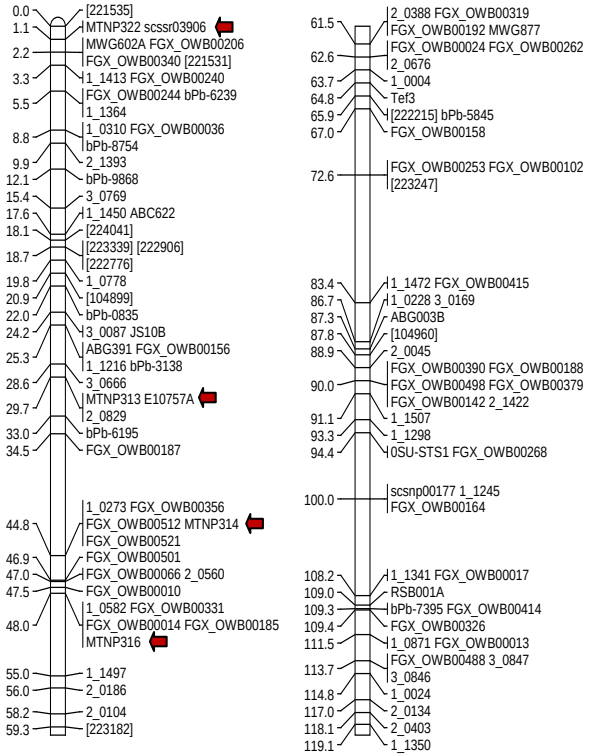
3H


0.0 [221563]
ABC172 FGX_OWB00228
FGX_OWB00303 3_0135
1.1 FGX_OWB00442 FGX_OWB00468
[221492] FGX_OWB00058
[221355]
1.5 FGX_OWB00489
2.2 3_0055
3.3 2_1500
4.3 3_1341
5.4 3_0229
FGX_OWB00369 FGX_OWB00270
6.5 bPb-0164
8.7 [222092]
10.9 bPb-4387 bPb-4169
12.0 3_1388 FGX_OWB00384
FGX_OWB00205
13.1 ABC805 2_0605
15.3 1_0935 FGX_OWB00462
FGX_OWB00518 FGX_OWB00513
18.6 bPb-0200 FGX_OWB00281
20.7 FGX_OWB00139
20.8 FGX_OWB00251 FGX_OWB00493
bPb-4456
21.9 2_0421
FGX_OWB00169 FGX_OWB00081
25.2 bPb-7413 [222583]
2_1531
26.3 3_1161
FGX_OWB00097 bPb-3899
28.5 FGX_OWB00040 FGX_OWB00126
FGX_OWB00137 FGX_OWB00392
bPb-2420
29.6 FGX_OWB00257 MTNP318
FGX_OWB00163
30.7 FGX_OWB00008 bPb-5129
36.4 3_0137 MWG883
FGX_OWB00033
37.5 FGX_OWB00272
FGX_OWB00101 FGX_OWB00250
38.6 bPb-4830
40.8 2_0085 bPb-5796
bPb-8506
41.9 scind02281
48.8 FGX_OWB00502 3_0092
49.2 FGX_OWB00345
49.5 FGX_OWB00510
49.8 3_0973
52.0 1_0842
53.1 2_0662 1_1141
55.3 3_0081
56.4 3_0507
57.5 2_0413 3_1238
58.6 MTNP321
63.1 FGX_OWB00180 3_0274
64.2 [223788] 1_0918
66.4 bPb-9110
67.5 1_1330
72.0 bPb-4209 bPb-3630
73.0 3_0928 scsnp00940
74.1 1_0584
76.3 3_1329
77.4 3_0375
78.5 2_1277
81.8 FGX_OWB00111 FGX_OWB00342
bPb-9336 2_1081
82.9 [223016] 3_0119
85.1 2_1493 FGX_OWB00292
86.2 1_0662
91.9 FGX_OWB00204 2_1381
2_1083
93.0 [221404]
94.1 3_0640
95.2 FGX_OWB00230 3_0090
99.6 3_1367 FGX_OWB00015
FGX_OWB00043 FGX_OWB00117
104.1 1_1021 FGX_OWB00018
106.3 2_0659
107.4 3_0325
109.6 3_0663
115.3 Bmag0225
116.4 23
119.7 FGX_OWB00110 1_1517
123.1 2_0115
FGX_OWB00423 scssr25691
125.3 FGX_OWB00161 [223370]
1_1454
127.5 3_1346
128.6 1_0683
129.6 2_0694 1_0452
130.7 3_0754
130.9 FGX_OWB00470
131.2 FGX_OWB00082
131.5 FGX_OWB00267
131.8 1_1394
132.9 2_1305
136.2 1_1284 FGX_OWB00527
137.3 1_1191
138.4 DsT-67
141.7 2_0325 FGX_OWB00130
142.8 bPb-6491
143.9 2_0444
3_1502 FGX_OWB00416
145.0 Bmac0209 FGX_OWB00068
2_1062 FGX_OWB00300
145.1 FGX_OWB00399
147.2 3_1008 FGX_OWB00030
FGX_OWB00429
148.3 2_0866
149.4 3_0680 FGX_OWB00297
alm
150.5 3_0923
151.6 3_0721
152.6 MTNP304 FGX_OWB00445
FGX_OWB00237 FGX_OWB00380
153.7 MTNP302

4H


0.0 2_1117
0.7 3_1324
4.5 MWG634
6.0 FGX_OWB00001 FGX_OWB00432
MTNP311
10.7 MTNP303
11.4 1_0490
15.2 FGX_OWB00440
15.9 MTNP315
17.4 1_0223 MTNP305
FGX_OWB00219 [104902]
18.1 1_0221
18.8 1_1199
19.6 1_1136
20.3 2_1458
21.0 1_0626
CDO542 FGX_OWB00232
21.7 2_1418
22.1 scsnp01648
22.5 CDO122
23.9 [222439] FGX_OWB00291
24.7 3_1164
FGX_OWB00284 3_1439
25.4 FGX_OWB00053
26.9 1_0171 FGX_OWB00119
27.6 bPb-6611
29.1 3_0187 scssr20569
31.3 1_0668
32.8 2_1490
FGX_OWB00279 FGX_OWB00330
FGX_OWB00398 FGX_OWB00378
35.0 scind04312a 3_1156
1_0577 FGX_OWB00052
35.7 3_0427
FGX_OWB00307 scind01728
36.4 1_0881 2_0610
FGX_OWB00358 Tef2
36.8 FGX_OWB00134
37.2 FGX_OWB00222
39.4 1_0086
40.9 1_1207 FGX_OWB00225
41.6 bPb-6259
42.3 Bmag0353 2_0098
43.0 3_0455
43.8 1_0606
46.8 scind10455 bPb-4214
47.5 FGX_OWB00078
48.2 bPb-8013
48.9 3_1362
1_0309 FGX_OWB00341
50.4 2_0207
51.1 FGX_OWB00155
52.6 2_1332
FGX_OWB00062 1_0170
54.1 scssr14079
54.8 ABG472 bPb-6096
55.5 FGX_OWB00406 1_0670
bPb-0610 2_0765
56.3 GBM1059
58.5 3_0138
60.7 2_0732
61.4 3_0142
62.2 1_0785
63.6 Ebmac0701
FGX_OWB00203 FGX_OWB00160
FGX_OWB00413 MWG652B
64.4 3_0117 FGX_OWB00050
FGX_OWB00029
FGX_OWB00382 FGX_OWB00109
1_1139 FGX_OWB00224
65.8 FGX_OWB00246 FGX_OWB00482
FGX_OWB00321
68.1 [223032] GBM1048
FGX_OWB00323
69.5 FGX_OWB00112 1_1194
MWGO77
70.3 3_0385
72.5 bPb-2677
73.2 bPb-1040
73.9 MTNP317
74.7 1_0697
75.4 2_0224
76.1 2_0760
76.8 3_0888 bPb-3468
77.2 [223334]
77.6 3_0020
78.3 3_1422
83.8 ABG601 bPb-9668

5H


0.0 [221535]
1.1 MTNP322 scssr03906
MWG602A FGX_OWB00206
2.2 FGX_OWB00340 [221531]
3.3 1_1413 FGX_OWB00240
5.5 FGX_OWB00244 bPb-6239
1_1364
8.8 1_0310 FGX_OWB00036
bPb-8754
9.9 2_1393
12.1 bPb-9868
15.4 3_0769
17.6 1_1450 ABC622
18.1 [224041]
18.7 [223339] [222906]
[222776]
19.8 1_0778
20.9 [104899]
22.0 bPb-0835
24.2 3_0087 JS10B
25.3 ABG391 FGX_OWB00156
1_1216 bPb-3138
28.6 3_0666
29.7 MTNP313 E10757A
2_0829
33.0 bPb-6195
34.5 FGX_OWB00187
44.8 1_0273 FGX_OWB00356
FGX_OWB00512 MTNP314
FGX_OWB00521
46.9 FGX_OWB00501
47.0 FGX_OWB00066 2_0560
47.5 FGX_OWB00010
48.0 1_0582 FGX_OWB00331
FGX_OWB00014 FGX_OWB00185
MTNP316
55.0 1_1497
56.0 2_0186
58.2 2_0104
59.3 [223182]
61.5 2_0388 FGX_OWB00319
FGX_OWB00192 MWG877
62.6 FGX_OWB00024 FGX_OWB00262
2_0676
63.7 1_0004
64.8 Tef3
65.9 [222215] bPb-5845
67.0 FGX_OWB00158
72.6 FGX_OWB00253 FGX_OWB00102
[223247]
83.4 1_1472 FGX_OWB00415
86.7 1_0228 3_0169
87.3 ABG003B
87.8 [104960]
88.9 2_0045
FGX_OWB00390 FGX_OWB00188
90.0 FGX_OWB00498 FGX_OWB00379
FGX_OWB00142 2_1422
91.1 1_1507
93.3 1_1298
94.4 0SU-STS1 FGX_OWB00268
100.0 scsnp00177 1_1245
FGX_OWB00164
108.2 1_1341 FGX_OWB00017
109.0 RSB001A
109.3 bPb-7395 FGX_OWB00414
109.4 FGX_OWB00326
111.5 1_0871 FGX_OWB00013
113.7 FGX_OWB00488 3_0847
3_0846
114.8 1_0024
117.0 2_0134
118.1 2_0403
119.1 1_1350
121.0 FGX_OWB00435
124.7 3_0182
125.8 bPb-5532
126.9 1_1134 FGX_OWB00266
FGX_OWB00184
128.0 FGX_OWB00190 1_0578
129.1 1_0930
130.1 1_0524
133.5 1_0674
138.0 1_1135 FGX_OWB00351
FGX_OWB00057 scsnp06144
141.3 2_1186
143.5 scssr15334
144.6 2_0736
scind16991 FGX_OWB00085
145.7 1_0167 2_0246
FGX_OWB00504
145.9 FGX_OWB00002
149.0 2_1128 FGX_OWB00084
ABC302 FGX_OWB00459
152.3 1_0305
154.5 2_0818
157.9 1_0953
159.0 3_0719 FGX_OWB00259
160.0 1_0548
166.9 FGX_OWB00159 [223146]
[222177] 1_0641
bPb-8987 [224132]
Ale
170.3 bPb-7627 FGX_OWB00039
FGX_OWB00038 scsnp04260
[223210]
171.4 3_1034 NRG045A
172.5 1_0725
173.5 1_1523
174.6 [221802] FGX_OWB00176
2_0501
175.7 3_1117 FGX_OWB00214
FGX_OWB00151
177.9 FGX_OWB00166 FGX_OWB00243
2_0396

7H

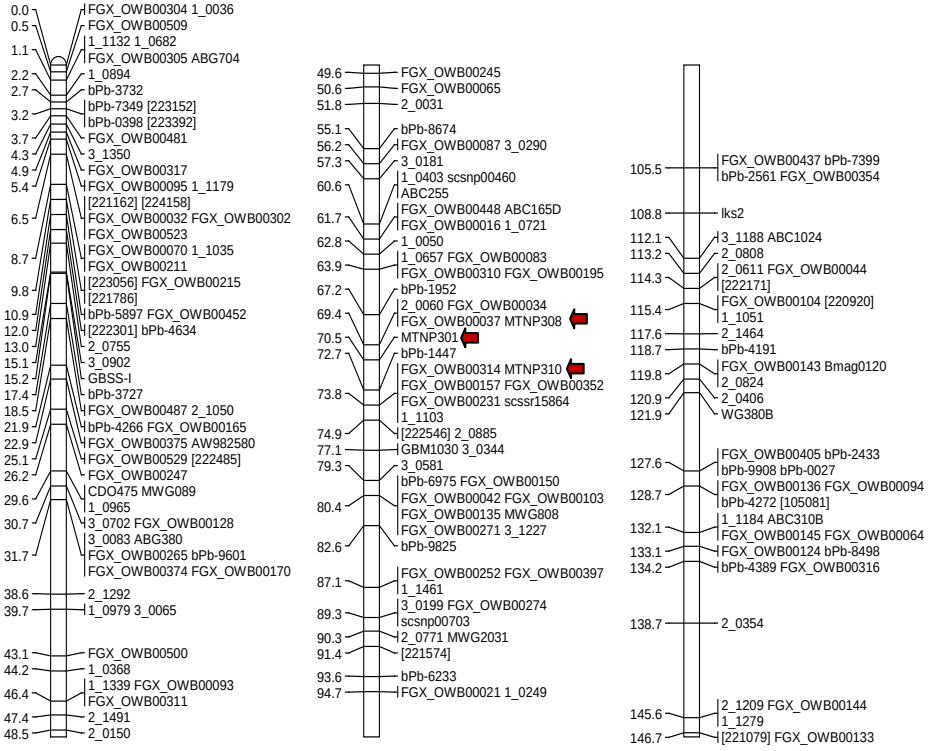

0.0 FGX_OWB00304 1_0036
0.5 FGX_OWB00509
1.1 1_1132 1_0682
FGX_OWB00305 ABG704
2.2 1_0894
2.7 bPb-3732
3.2 bPb-7349 [223152]
bPb-0398 [223392]
3.7 FGX_OWB00481
4.3 3_1350
4.9 FGX_OWB00317
5.4 FGX_OWB00095 1_1179
[221162] [224158]
6.5 FGX_OWB00032 FGX_OWB00302
FGX_OWB00523
8.7 FGX_OWB00070 1_1035
FGX_OWB00211
9.8 [223056] FGX_OWB00215
[221786]
10.9 bPb-5897 FGX_OWB00452
12.0 [222301] bPb-4634
13.0 2_0755
15.1 3_0902
15.2 GBSS-I
17.4 bPb-3727
18.5 FGX_OWB00487 2_1050
21.9 bPb-4266 FGX_OWB00165
22.9 FGX_OWB00375 AW982580
25.1 FGX_OWB00529 [222485]
26.2 FGX_OWB00247
29.6 CDO475 MWG089
1_0965
30.7 3_0702 FGX_OWB00128
31.7 3_0083 ABG380
FGX_OWB00265 bPb-9601
FGX_OWB00374 FGX_OWB00170
38.6 2_1292
39.7 1_0979 3_0065
43.1 FGX_OWB00500
44.2 1_0368
46.4 1_1339 FGX_OWB00093
FGX_OWB00311
47.4 2_1491
48.5 2_0150
49.6 FGX_OWB00245
50.6 FGX_OWB00065
51.8 2_0031
55.1 bPb-8674
56.2 FGX_OWB00087 3_0290
57.3 3_0181
1_0403 scsnp00460
60.6 ABC255
FGX_OWB00448 ABC165D
61.7 FGX_OWB00016 1_0721
62.8 1_0050
63.9 1_0657 FGX_OWB00083
FGX_OWB00310 FGX_OWB00195
67.2 bPb-1952
69.4 2_0060 FGX_OWB00034
FGX_OWB00037 MTNP308
70.5 MTNP301
72.7 bPb-1447
FGX_OWB00314 MTNP310
73.8 FGX_OWB00157 FGX_OWB00352
FGX_OWB00231 scssr15864
1_1103
74.9 [222546] 2_0885
77.1 GBM1030 3_0344
79.3 3_0581
bPb-6975 FGX_OWB00150
80.4 FGX_OWB00042 FGX_OWB00103
FGX_OWB00135 MWG808
FGX_OWB00271 3_1227
82.6 bPb-9825
87.1 FGX_OWB00252 FGX_OWB00397
1_1461
89.3 3_0199 FGX_OWB00274
scsnp00703
90.3 2_0771 MWG2031
91.4 [221574]
93.6 bPb-6233
94.7 FGX_OWB00021 1_0249
105.5 FGX_OWB00437 bPb-7399
bPb-2561 FGX_OWB00354
108.8 lks2
112.1 3_1188 ABC1024
113.2 2_0808
2_0611 FGX_OWB00044
114.3 [222171]
115.4 FGX_OWB00104 [220920]
1_1051
117.6 2_1464
118.7 bPb-4191
119.8 FGX_OWB00143 Bmag0120
2_0824
120.9 2_0406
121.9 WG380B
127.6 FGX_OWB00405 bPb-2433
bPb-9908 bPb-0027
128.7 FGX_OWB00136 FGX_OWB00094
bPb-4272 [105081]
132.1 1_1184 ABC310B
FGX_OWB00145 FGX_OWB00064
133.1 FGX_OWB00124 bPb-8498
134.2 bPb-4389 FGX_OWB00316
138.7 2_0354
145.6 2_1209 FGX_OWB00144
1_1279
146.7 [221079] FGX_OWB00133

Figure 4.4: Confirmation of mapped position of MTNP lines (MTNP 301 and MTNP 310) with genetic linkage map constructed using Joinmap4. Red arrows indicate the position of MTNP lines.

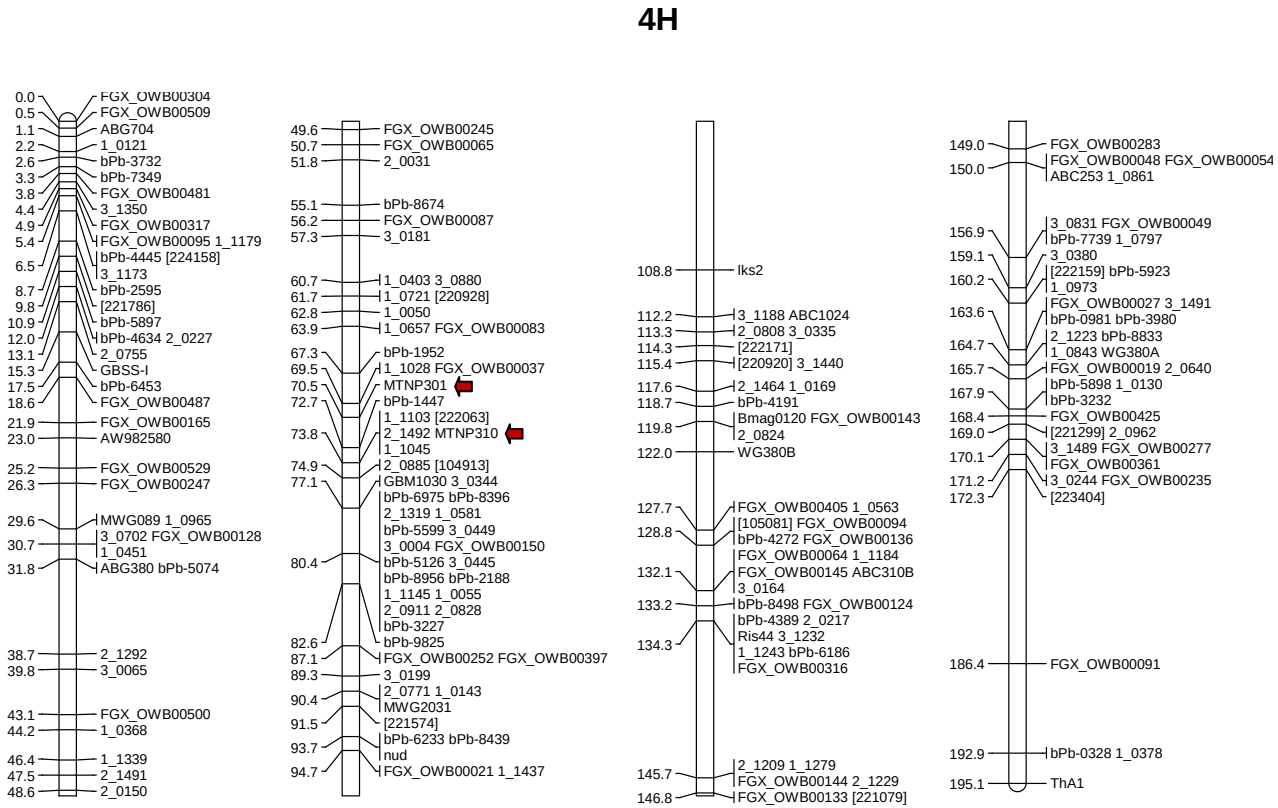
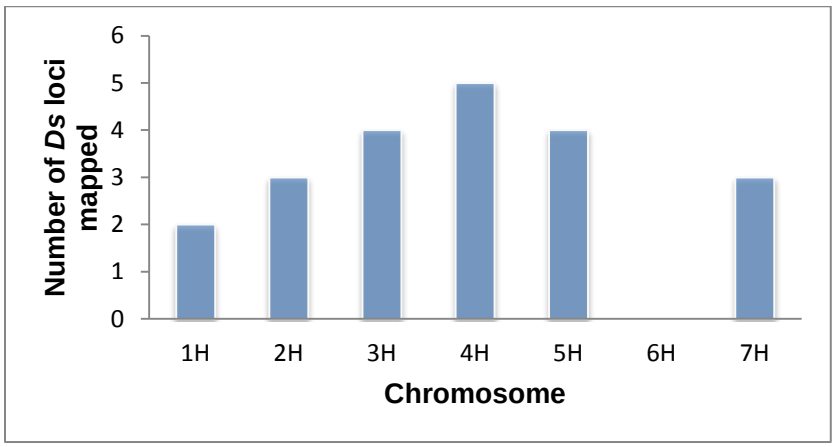


Figure 4.5: Frequency of MTNP lines location across barley chromosomes**.**



4.5 Discussion

Malting quality of barley is an important industrial characteristic, but a difficult trait for genetic studies. Our goal to dissect the important malting quality QTL2 using transposon-based mutagenesis relies on the fact that the majority of *DsT* (*Ds* tranposant) lines transpose into nearby linked positions.

The major goal of this study was to genetically locate *Ds* transposon insertions across the barley chromosomes. We had generated 10 *Ds* insertions in our previous work; Singh et al., (2012), in order to saturate the malting quality QTL2. In the present study, we mapped 22 new sequences to the barley linkage map.

4.5.1 Characterization of flanking sequences

Various methods were employed to sequence the flanking region of a transposon insertion including iPCR, TAIL PCR, Adapter ligation etc., but none of them proved to be efficient for all the new *Ds* insertion lines generated. Therefore, we employed all these techniques to generate the maximum number of sequences flanking the insertion. In the present study, we introduced adapter based ligation method where iPCR and TAIL PCR were not successful. The flanking sequences we generated perform a variety of functions, and the *Ds* mutants can be used for their further investigation. Some of the important ones areincluded: MTNP-311 which has homology with *Hordeum vulgare* serine/threonine kinase-like protein, belongs to protein kinase superfamily, and functions in ATP binding and enzyme regulator. *Ds* mutants in this gene may help to characterize the encoded protein. MTNP-318, which has similarity with the *Lks2* gene (putative short internodes family transcription factor) and functions in regulation of awn elongation and pistil morphology in barley. This gene might have future relevance in alleviating lodging and grain yield. Corresponding mutants generated from other *Ds* insertions can be used to characterize gene function or other molecular aspects. In addition the *Ds* insertions line MTNP-316 show some sequence similarity with an EST expressed at malting stage of barley. This can be a putative candidate gene to study malting related traits.

4.5.2 Polymorphisms and linkage map construction

We detected polymorphisms using a sequenced based approach and gel electrophoresis. The majority of flanking sequences had either an SNP or Indel polymorphism. However, we did not detect any polymorphism in five MTNP lines, in any mapping population. These five were subsequently mapped using the barley sequencing database. Seven MTNP lines detected SNP polymorphism in OWB DH population, two in the Steptoe X Morex population and two in the Dicktoo X Morex mapping population. Likewise, three were mapped by assaying for indels in the OWB DH population and two in the Steptoe X Morex population. Although there are many strategies to score an SNP marker in mapping population, but we preferred a restriction-digestion based assay called CAPs as demonstrated by Konieczny and Ausubel (1993).

A linkage map generated from an OWB population in 2011 (Chutimanitsakun et al., 2011) was used in this study. The inferred linkage map positions of the polymorphic loci in the Steptoe X Morex or Dicktoo X Morex mapping population were assigned based on the presence of markers common to the OWB population in the map. MTNP lines were mapped across the seven chromosome of barley; however, none was located on chromosome 6H. The majority of *Ds* loci were mapped on chromosome 4H, with high frequency in the QTL2 region. Chromosome location of all mapped lines was confirmed using barley sequencing database. The approximate locations of the *Ds* insertions could also be determined using wheat deletion lines or the wheat: barley addition lines, but these approaches could only locate *Ds* lines to chromosome arm, or a sector of a chromosome arm, and lack the resolution necessary to assign *Ds* elements to specific barley linkage map.

4.5.3 Frequency of transposition

TNP-29 and TNP-79 lines that map close to malting quality QTLs were used as launch pads to generate new *Ds* transposants; therefore, we expect a higher frequency of *Ds* insertions into the QLTs. This anticipation was based on previous studies that suggest a high frequency of *Ds* insertion into nearby positions (Brown et al., 2015, Koprek et al., 2000; Kumar and fladung 2003).

We mapped 22 lines, of which five were located on chromosome 4H. This frequency is lower than in previous studies. However our work (Brown et al., 2015) with other lines, in which we mapped 200 *Ds* lines show a linked frequency of 80%, which is consistent with earlier studies of 75% transposition into linked sites in barley (Koprek et al., 2000). In *Arabidopsis* this frequency is about 50% (Raina et al., 2002), and in rice 62-67 % transpositions are linked (Upadhyaya et al., 2002). A likely reason of lower frequency in our results is due the limited number of insertions available for study, which prevented a rigorous statistical analysis of the nature of *Ds* transposition.

It is also evident, as in previous studies (Brown et al., 2015, Singh et al., 2006, Hehl and Baker 1989) that deletion or alteration of the 11 bp terminal repeats renders the *Ds* element unable to transpose in following generations. The state of 8-bp, direct-repeat footprints, immediately next to the TIRs also reveals a strong correlation between perfect TIRs and complete 8-bp duplications and between defective TIRs and imperfect 8-bp direct repeats. Generation of mutants as a result of deletions in terminal repeats and genic regions can be utilized to generate new alleles.

4.6 Conclusion

The principal objective of this study was to map *Ds* insertions (MTNP lines) in the barley genome using the OWB DH population to facilitate integration with other genomics resources in barley. We were interested in performing a preliminary characterization of the sites of insertion of the *Ds* elements and mapping them. We employed an adapter ligation technique in addition to iPCR and TAIL PCR methods to amplify flanking *Ds* sequences. All flanking sequences were mapped on barley chromosomes, with a high frequency of them in the QTL2 region. The overall goal of this project is to dissect the QTL2 malting region. Results from this study provide candidate genes that can be studied in future for malting traits.

CONNECTING STATEMENT BETWEEN CHAPTER IV & V

Generation and mapping of new *Ds* transposon insertions indicated the location of candidate malting genes in the QTL2 region. Therefore, we considered this large region of chromosome 4 worth investigating further. Whereas in chapter 4 we employed a transposon-based approach, our study reported in chapter 5 used synteny-based comparative genomic approach to further dissect the QTL2 region of chromosome 4 to examine the potential role of the genes harbored in this region in malting.

The QTL2 region of barley chromosome 4H was found to be collinear with rice chromosome 3L, from which we have retrieved over 100 genes. Further bioinformatic analysis helped us identify 24 candidate genes potentially involved in germination and malting. From among these candidate genes, we successfully identified *HvTLP8* gene using genomic/transcriptomic and biochemical assays. The HvTLP8 protein was found to interact with β-glucan indicating its potential role in the malting process.

I carried out all the experiments reported in chapter V. I also played a major role in designing the experimental set-up and wrote the first draft of the manuscript. The experimental studies used involved analysis based on comparative genomics, transcriptomic, proteomics, and biochemical analysis. My supervisor, Dr. Jaswinder Singh provided valuable discussions in experimental design and writing of the manuscript, and his NSERC-CRD grant provided funding.

Chapter V

Barley Thaumatin-Like Protein gene *HvTLP8* resides in QTL2 and its expression regulates the availability of β-glucan during grain germination

Surinder Singh and Jaswinder Singh*

*Corresponding Author: Dr. Jaswinder Singh

Plant Science Department, 21 111 Rue lakeshore

McGill University, Quebec, H9X 3V9, Canada

Email: jaswinder.singh@mcgill.ca

Phone: +1(514) 398-7906

Singh S, Singh J (2015) Barley Thaumatin-Like Protein gene *HvTLP8* resides in QTL2 and its expression regulates the availability of β-glucan during grain germination

5.1 Abstract

Barley is a key ingredient in the malting and brewing industry; therefore, gene discovery in relation to malting quality has industrial importance. More than 250 quantitative trait loci (QTL) have been associated with 19 malting quality traits in barley. However, only a few genes have been characterized for their roles in malting quality. Chromosome 4H of barley contains a major malting QTL, QTL2, near the telomeric region, which influences malt extract percentage, diastatic power, and malt glucan content . However, specific roles of genes in QTL2 in determining specific malting traits are presently unclear. Co-linearity between different genomes has been used for identifying and characterizing genes in different crop species. Comparative genomics of barley and rice chromosomes has established that the terminal region of barley chromosome 4HS is syntenic to rice chromosome 3L. This allowed us to dissect the QTL2 region using rice-barley microsynteny. From a set of 24 ESTs (expressed sequence tags), expressed in seeds and at malting stages, a candidate gene (*HvTLP8)* was found to be polymorphic and to affect malting traits. Gene expression and protein profiles of *HvTLP8* were subsequently studied in different malt/feed barley varieties using qPCR and immunoblotting. Results obtained allowed us to conclude that HvTLP8 protein binds insoluble β-glucan present in barley seeds and, thereby, lowers its availability in the malting process. Our findings suggest that higher amounts of HvTLP8 protein can minimize problems encountered during the beer lautering and filtration processes.

Key words: *Hordeum vulgare*, Malting, Differential gene expression, Quantitative Trait Loci, Comparative genomics, Thaumatin like protein

5.2 Introduction

The genes coding for Thaumatin-like proteins (TLPs), also designated as PR-5 (pathogenesis-related-5), represent a complex gene family involved in a broad range of defense and developmental processes in plants, fungi, *Caenorhabditis elegans*, insects, mites and vertebrates (Brandazza et al., 2004). TLPs share sequence similarity with thaumatin, a sweet-tasting protein originally found in the fruit of the shrub *Thaumatococcus daniellii* (Vanderwe and Loewe 1972). In plants, TLPs are members of an inducible group of proteins that includes 17 families of pathogenesis-related proteins termed PR-1 to PR-17. Their synthesis in plants is triggered mainly in response to biotic and abiotic stress, but is also developmentally regulated, particularly during fruit ripening (Fils-Lycaon 1996). TLPs may also be constitutively present in varoius plant organs, including grains.

TLPs are highly conserved, possessing molecular mass of 21- 26 kD, with anti-parallel beta sheets and eight disulfide bridges formed by 16 conserved cysteine residues (Liu et al., 2010). These bridges are required for correct folding and stability during high temperature or low pH (Fierens et al., 2009). Plant TLPs contain an acidic cleft, which acts as a receptor for their binding activity (Batalia et al., 1996, Min et al., 2004). During the course of evolution, TLPs have adapted a great functional diversification, possibly due to their interaction with numerous ligands such as β- glucan, actin, ice crystals, and fungal proteins. Because they can bind ice crystals, some of them function as effective antifreeze proteins. (Griffith et al., 2005) Some of the essential functions in plants include antifungal activity (Velazhahan et al. 1999), protection against osmotic stress (Kononowicz et al., 1992), freezing tolerance (Chun and Griffith, 1998), binding to proteins such as actin, viral CMV-1 protein, yeast glycoproteins and G-protein-coupled receptors (GPCR) or to hormones such as cytokinins (Liu 2010), β-glucan binding (Trudel et al. 1998) glucanase activity (Grenier et al. 1999) and xylanase inhibition (Fierens et al., 2007). TLPs are expressed in grains during germinating (Hejgaard 1991, Osmond 2001), but their role in pre- and/or post- germination processes such as malting and fermentation is unknown.

Barley, being an essential ingredient in malting, has been a target of investigation throughout the 5000-year-old history of brewing process. Improving the genetics and biochemistry of barley for superior malting quality has been a key objective of barley breeders. Generally, varieties possessing characteristics like high content of starch-degrading enzymes and lower protein content have been used as malt varieties and those lacking such traits, as feed varieties (Molina-cano et al., 1997). A low β-glucan and protein content have been considered desirable traits for malting (Zang et al., 2001) Excessive amounts of β-glucan and protein lead to high viscosity of malt extract, diminished rate of wort filtration and haze formation in beer (McClear 1985). Manipulation of malting quality traits (malt extract, diastatic power, α-amylase, malt β-glucan, and malt β- glucanase) is challenging for geneticists and breeders due to complex inheritance and difficulty in evaluation of these quantitative traits. Measuring malting quality is an expensive and laborious process, limiting its use in barley cultivar development and contributing to the challenges in breeding barley for malting quality (Hayes et al., 2003; Gao et al., 2004).

Genetic studies of malting traits involve studying QTLs, and more than 250 QTLs have been located on barley chromosomes (Wei et al., 2009). Among these, QTL2 is a major determinant of malt extract percentage, α-amylase activity, diastatic power, and malt glucan content. QTL2 is is a segment of 15.8 cM in size and is located near the telomeric end of the short arm of chromosome 4H (Han et al., 1997; Hayes et al., 1993; Gao et al., 2004). Here, our studies are directed towards dissecting QTL2 to identify genes affecting malting traits. We describe a novel function of *HvTLP8,* a gene located in the QTL2 region that contributes to superior malting quality. The results of our study suggest that *HvTLP8* contains a carbohydrate-binding domain which captures β-glucan with tight affinity and lowers its amount in the malt for downstream brewing process. We suggest that *HvTLP8* gene confers a superior malting quality.

5.3 Material and methods

5.3.1 Search for candidate genes with potential role in malting

A comparative approach using flanking EST markers from the QTL2 region in the barley EST map (http://harvest.ucr.edu/) and their corresponding region on rice 3L chromosome using Rice 7 map (rice genome browser of the rice genome database, www.tigr.org) (Kawahara et al., 2013) was used. All genes and gene models predicted in the rice syntenic region were analyzed for candidates in malting. Initial candidates (24) were selected from more than 100 genes found in the QTL2 corresponding region of rice, based on their functional homology with ESTs expressed at malting stages in seed. Specific primers were designed from these 24 candidates and were used to amplify genomic sequences from commonly used malting and feed varieties. Primer sequence MSB12 corresponding to *HvTLP8* gene was selected as it showed differential polymorphism between malting and feed varieties. The full *HvTLP8* gene sequence was retrieved using localization of Morex WGS contigs in the integrated physical and genetic map of barley (IBGSC, 2012; URL: ftp://ftpmips.helmholtz-muenchen.de /plants/barley /public data/).

Conserved carbohydrate-binding domain (glycoside hydrolase family 64) on the protein structure, among different species were obtained using the NCBI conserved domain architecture retrieval tool and motif using Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) (Sigrist et al., 2013). Barley sequence database was used to download all the contigs similar to *HvTLP8,* in a malting (Morex) variety and feed (Bowman) variety. Each of these contigs was further compared among themselves, for highest homology. More than one hit in contigs were assembled to a consensus sequences. Homologous sequences obtained from Morex and Bowman were used to predict protein secondary structure, using online tool available at http://www.sbg.bio.ic.ac.uk/phyre2 (Kelley et al., 2009) Probable pockets in the protein structure were determined using web-based tool http://fpocket.sourceforge.net/ (Le Guilloux et al., 2009).

5.3.2 RNA isolation, cDNA synthesis and quantitative RT- PCR

Six registered malting and feed barley varieties (obtained from PGRC, Saskatoon) were used. For RNA isolation, mature barley grains were surface sterilized with 70% ethanol, 4% NaOCl, and germinated in the dark on wetted filter paper at 21°C for 16, 48 and 96 hr. RNA extraction was carried out using a method modified from Li and Trick 2005, and optimized for seeds containing high amounts of starch. For cDNA synthesis, a 500ng sample of DNase-treated RNA was reverse transcribed using an iScript cDNA synthesis kit (Bio- Rad, Hercules, CA). The transcribed cDNA was diluted with an equal amount of sterile water for analysis.

For qRT-PCR, two biological replicates and three technical replicates for each sample, along with two negative controls were used. Relative expression with two reference genes (Beta-Actin and GAPDH) with Brilliant III SYBR Green QPCR master mix (Agilent Technologies) was conducted, following the manufacturer's recommendations. Specific primers were designed for RT-PCR (F: TTCCGTCCTGCTCCCAATC, R: TGCAGC AGTACCTGTCCTC) and qRT-PCR (F: GTGCCCATGTCGTTCGTC, R: TGCAGCA GTACCTGTCCTC). Amplification was performed in a 20 µL reaction mixture containing 160 nmol of each primer, 1X Brilliant III SYBR Green QPCR master mix, 15 µM ROX reference dye and 0.3 µL cDNA template. The amplification conditions were 95ºC for 10 min (hot start), followed by 30 cycles at 94ºC for 30 s, 58ºC for 30 s and 72ºC for 30 s. The fluorescence reading was taken at 72ºC at the end of the elongation cycle. The relative expression ratios of the target genes versus reference genes were calculated with the equation developed by Zhao and Fernald, based on crossing point (CP) and efficiency obtained for each sample amplified with the reference genes and the target genes using the equation: R0 = 1/(1+E)CT, where R0 is the initial template concentration, E is the efficiency in the exponential phase and CT is the cycle number at threshold. Analysis was performed using the web-based software Real-Time PCR Miner (http://www.miner.ewindup.info/version2) (Zhao et al., 2005).

5.3.3 Protein purification, SDS PAGE, immuno-blotting and β-glucan content

Barley grains (1g) from each variety were surface sterilized, steeped and germinated in the dark at 21± 2º C under aseptic conditions in a Petri-dish with autoclaved Watman filter paper. Germinated grains were ground with 10 ml of 50 mM sodium phosphate buffer, pH 7.0, and centrifuged at 15,000g for 15 min at 4º C. The supernatant was subjected to ammonium sulfate fractionation and the pellet obtained at 30-60% saturation was re-dissolved in 10 ml of 50 mM sodium acetate buffer, pH 5.0. The Bradford method was used for protein quantification.

For polyacrylamide gel electrophoresis (PAGE), proteins were separated by electrophoresis through 5% (w/v) polyacrylamide stacking and 15% resolving gel, using Mini-Protein gel assembly (BioRad). Gels were stained with Coomassie Brilliant Blue R (BioRad) in 25% (v/v) ethanol and 7% (v/v) glacial acetic acid, and destained in the same solution without dye. For Western analysis, proteins were transferred to PVDF membrane filters (Millipore, USA), blocked with 5% nonfat dry milk for 1 hr, and rinsed three times with TBS-T (with 0.05% Tween-20) buffer. The membrane was incubated with anti-thaumatin antibody (abcam) in 1:1000 overnight at 4^0C, washed 3 times in TBS-T and incubated further with anti-chicken IgY for 1 hr at room temperature, followed by 3 TBS-T washes. The blot was developed with 1200 µl of substrate (Clarity™ Western ECL substrate, Bio-Rad). Unbound (normal) proteins and bound proteins, with β-glucan were analysed on PAGE and transferred to blot. For bound proteins, pellet obtained after binding between HvTLP8 and β-glucan was boiled in SDS buffer and loaded to gel.

The amount of β-glucan at different stages of germination was determined using β-glucan kit from Megazyme (Ireland).

5.3.4 Binding assay, ELISA and glycosylation

For binding assays purified barley TLP proteins (5 nmol) were incubated with 5 mg of insoluble β-glucan in 50 mM sodium acetate buffer, pH 5.0, in a volume of 0.5 ml. The protein-polysaccharide mixture was incubated at 25º C for 1 hr, on an orbital shaker,

and centrifuged thereafter at 1000 g for 3 min. The supernatant was analyzed for residual protein using an ELISA-based detection system, developed in our lab. Commercially available thaumatin proteins (Sigma, St. Louis, USA) were used as a positive control and to generate a standard curve. Proteins adsorption was estimated by subtraction of residual protein in the supernatant from the values obtained in assays conducted without polysaccharide. To carry out ELISA with different redox potential, ammonium sulphate fraction of protein was treated with following sulfhydrl reagents before binding with β-glucan: 5mM Dithiothreitol (DTT), 0.5mM DTT, 10mM reduced glutathione and 10 mM β-mercaptoethanol. Subsequently binding and next steps of ELISA were performed as per standard protocol provided with commercial ELISA kit (Sigma, St. Louis, USA).

Glycosylation analysis was performed using Pro-Q ®Emerald 300 Glycoprotein Gel Stain Kit (life technologies). Normal and bound proteins were transferred from an SDS-PAGE gel to a PVDF membrane. The membrane was subsequently stained Pro-Q ®Emerald 300 Glycoprotein Gel Stain Kit following manufacture's guidelines.

5.4 Results

5.4.1 Barley QTL2 maintains synteny with rice 3L

We investigated micro-synteny of the malting QTL2 region (chromosome 4H) of barley, with chromosome 3L of rice. QTL2 spans a region of 15.2 cM in the telomeric region of chromosome 4H, and the syntenic region of rice has 1.795 Mbp (25328757bp – 27123813 bp) coverage on chromosome 3L (Figure 5.1). Nearly 80% of markers mapped in the QTL2 of barley maintained co-linearity on the chromosome 3L of rice. In the rice QTL2 syntenic region, >100 genes/predicted genes were detected. Each gene was analysed by comparing homology against various EST databases (HarvEST, IPK Crop EST Database). Twenty-four candidate genes were selected based on EST expression at germination stages with a potential role in determining malting-related traits. Putative functions of the candidate genes were assigned with BLASTx analysis. Table 5.1 shows BLASTx homology, map location and putative function of selected genes.

5.4.2 *HvTLP8* structure and bioinformatic analysis

HvTLP8 encodes a 233 kDa protein with a conserved carbohydrate-binding domain (GH64-TLP-SF). In barley, 8 types of TLPs (HvTLP1 to HvTLP8) have been reported. Phylogenetically TLPs 1-4 form one clade and TLPs 4-8 form another (Figure 5.2). Mature *HvTLP8* mRNA contains 2345 bp (Figure 5.3a), with a 776 bp single exon as the coding region and the presence of a carbohydrate-binding domain within this region. A 24-bp unique structural motif, forming a beta hairpin may function in carbohydrate-binding activities. The coding region is flanked by a 444 bp 5' UTR and 1104 bp 3' UTR and a 5' cap and 3' poly A tail. The protein structure of the HvTLP8 displays a 233 a/a protein with eight disulfide linkages, 4 helices and 3 β-hairpins. A β-hairpin at the site of a structural motif forms an acidic cleft (pocket) that might function as the polysaccharide binding site (Figure 5.3b).

The barley whole genome sequence database provides sequence information for malt (Morex) and feed (Bowman) varieties. We analyzed all *HvTLP8* homology contigs retrieved from the database (Table 5.2). Barley TLPs (1-8) have a high degree of homology among themselves; however *HvTLP8* possess highest homology with a single contig from malt variety, and three contigs from feed variety, as shown in table 5.2. Consensus sequence from the Bowman contigs was determined, to obtain full length *HvTLP8*. Translated product of *HvTLP8* resulted in a product possessing a single amino acid change between Morex and Bowman variety (Figure 5.4c). Further analysing the predicted secondary structure using http://www.sbg.bio.ic.ac.uk/phyre2 (Kelley et al., 2009) resulted in a difference of α-helix (amino acid position 178) and β-strand (amino acid position 117 and 155) among cultivars as shown in figure 5.4a and 5.4b. The thaumatin family signature i.e G-x-[GF]-x-C-x-T-[GA]-D-C-x(1,2)-[GQ]-x(2,3)-C, which is characteristic of all TLPs, was present in amino acid sequence of Morex and bowman variety. Figure 5.4 shows the presence of motif (highlighted), which may play role in folding and binding carbohydrate.

5.4.3 *HvTLP8* is highly expressed in malting barley varieties and is related to levels of β-glucan.

Messenger RNA levels for candidate gene in barley malting and feed varieties were compared via semi-quantitative RT-PCR. RNA samples extracted from different stages of barley germination were used as templates. The *HvTLP8* genes differentially expressed across the varieties, at different times during germination process with higher levels of transcripts in malting and lower in feed varieties (figure 5.5a). As germination proceeded in time, *HvTLP8* was found to be up-regulated in malting varieties and down-regulated in feed varieties. *HvTLP8* expression during initial 16 h of germination was higher and subsequently decreased in 48 h. Differential messenger RNA level of *HvTLP8* observed by RT-PCR at 16h of germination was further confirmed by qRT-PCR. Altogether, malting varieties had an approximate three fold higher gene expression than feed varieties (Figure 5.5).

5.4.4 Molecular and biochemical assays for quantification of β-glucan binding

As described above in section 5.3.2, proteins before and after binding with β-glucan were analysed on a gel and immunoblot. Based on intensity of bands in western blot analysis, a higher amount of TLP proteins was observed in normal extracts (non-bound) of all six barley varieties. Overall, malting varieties displayed a greater quantity of TLP proteins than feed varieties. In case of feed varieties, low or negligible binding with β-glucan was observed with TLP antibodies (Figure 5.7c). In addition immunoblotting of bound and unbound proteins from malting and feed barley varieties revealed protein bands of slightly higher molecular mass in malting than feed varieties (Figure 5.7c), indicating glycosylation though β-glucan binding. This carbohydrate (β-glucan) - protein (HvTLP) interaction was validated with a glycosylation assay. HvTLP8 protein bands after binding had high intensity fluorescence in malting varieties, when observed under UV light (Figure 5.7a & 5.7b). No signal was observed in unbounded control proteins either from malting or from feed varieties.

To infer the binding competence of barley HvTLP8 with β-glucan, ELISA was performed. Indirect ELISA provided a quantitative assay, to determine the level of

binding between β-glucan and HvTLP8. Figure 5.8 depicts a graphical representation of the ELISA results, with various treatments under reducing conditions. We observed higher binding with β-glucan in malting varieties compared to feed, carried in two biological and three technical replicates. Statistically, 44 to 65 % of β-glucan binds with HvTLP8 in malting varieties, as compared to 9 to 13% in feed varieties. The effect of different sulfhydryl reducing agents on binding capacity of HvTLP8, inferred that reduced glutathione, DTT and β-mercaptoethanol effectively reduce the binding of β-glucan to HvTLP8. From these redox reagents, reduced glutathione effectively prevents the binding of HvTLP8, to a negligible level. These results indicate a probable role of disulfide bonds during HvTLP8 and β-glucan binding through thiol-disulfide exchange reaction.

Table 5.1: Table summarizing candidate genes obtained using synteny comparison of the barley QTL2 region with rice 3L. It also includes information about the tissue where the EST is expressed, their homology with BLASTx and map location. Map position was confirmed with IPK barley database (http://webblast.ipk-gatersleben.de/barley/)

Candidate marker	Expression profile	Homology (BLASTx)	E-Value	Chromosome location
MSB1	Seed/Malting	Amino acid permease family protein	1.00E-154	4HS
MSB2	Seed/Malting	Predicted protein: not characterized	3.00E-180	4HS
MSB3	Seed/Malting	High affinity cationic amino acid transporter	5.00E-88	4HS
MSB4	Seed/ Malting	Glucan endo-1,3-beta-glucosidase	2.00E-134	4HS
MSB5	Embryo/Malting	Isopropylmalate dehydrogenase	3.00E-62	2H/4HS
MSB6	Seed/Malting	High affinity cationic amino acid transporter 1	7.00E-113	4HS
MSB7	Seed/ Malting	Beta – tubulin	0.00E+00	5H/4HS
MSB8	Seed/ Malting	Beta – tubulin	0.00E+00	4HS
MSB9	Endosperm/ malting	Guanine nucleotide exchange factor in Golgi transport N-terminal	5.00E-93	4HS
MSB10	Embryo / Malting	Embryo globulin, storage protein	6.00E-87	4HS
MSB11	Seed/Malting	Putative senescence- associated protein	3.00E-97	4HS
MSB12	Endosperm/ Malting	Thaumatin-like protein	1.00E-113	4HS
MSB13	Embryo, Seed/ Malting	40S ribosomal protein	1.00E-47	4HL
MSB14	Embryo, Scutellum/ Malting	Predicted: acidic leucine-rich nuclear phosphoprotein	4.00E-65	4HS
MSB15	Endosperm/Malting	Low temperature-responsive RNA-binding protein	5.00E-54	4HS
MSB16	Endosperm/ malting	Putative: early-responsive to dehydration protein	5.00E-179	4HS
MSB17	Seed/ Malting	RING finger protein 5	5.00E-58	4HS
MSB18	Seed/ Malting	Predicted protein	5.00E-160	4HL

MSB19	Endosperm/Malting	Predicted: glucose-induced degradation protein	8.00E-123	4HS
MSB20	Endosperm/Malting	Benzothiadiazole-induced homeodomain protein	0.00E+00	4HS
MSB21	Seed/ Malting	Ubiquitin-conjugating enzyme	6.00E-101	5H
MSB22	Embryo, Endosperm/ Malting	Putative ubiquitin-conjugating enzyme E2	3.00E-85	4HS
MSB23	Seed	Peptide transporter	1.00E-54	4HS
MSB24	Embryo	TOM1-like protein	1.00E-02	4HS

Table 5.2: Identification of putative *HvTLP* genes from available genome sequence database of *Hordeum vulgare* var. morex (malt) and *Hordeum vulgare* var. bowman (feed). TLP signature for presence (Y), absence (N) or altered (A) amino acid sequence is mentioned.

TLP	Homology to the genome sequence of malting variety (morex) (%, e-value)	Homology to the genome sequence of feed variety (bowman) (%, e-value)	Chromosome location	TLP signature*	
				malt	feed
TLP1	morex_contig_1638271	bowman_contig_72751	7HL	N	A
TLP2	morex_contig_75328	bowman_contig_144387	7HL	A	N
TLP3	morex_contig_44624	bowman_contig_67894	5HS	Y	N
TLP4	morex_contig_144177	bowman_contig_17817	5HS	A	N
TLP5	morex_contig_12479	bowman_contig_64947	5HS	N	Y
TLP6	morex_contig_6783	bowman_contig_64947	5HS	Y	Y
TLP7	morex_contig_1580630	bowman_contig_880057	5HS	N	Y
TLP8	morex_contig_2553017	bowman_contig_804 bowman_contig_17147 bowman_contig_1029	4HS	Y	Y

*TLP signature: G-x-[GF]-x-C-x-T-[GA]-D-C-x(1,2)- [GQ]-x(2,3)-C

Figure 5.1: Figure depicts the conserved syntenic region between barley chromosome 4HS and rice chromosome 3L. A segment (0-15cM) on short arm of barley chromosome 4H represents QTL2 which is syntenic and maintains co-linearity with a region on chromosome 3L of rice. Figure designed using strudel software.

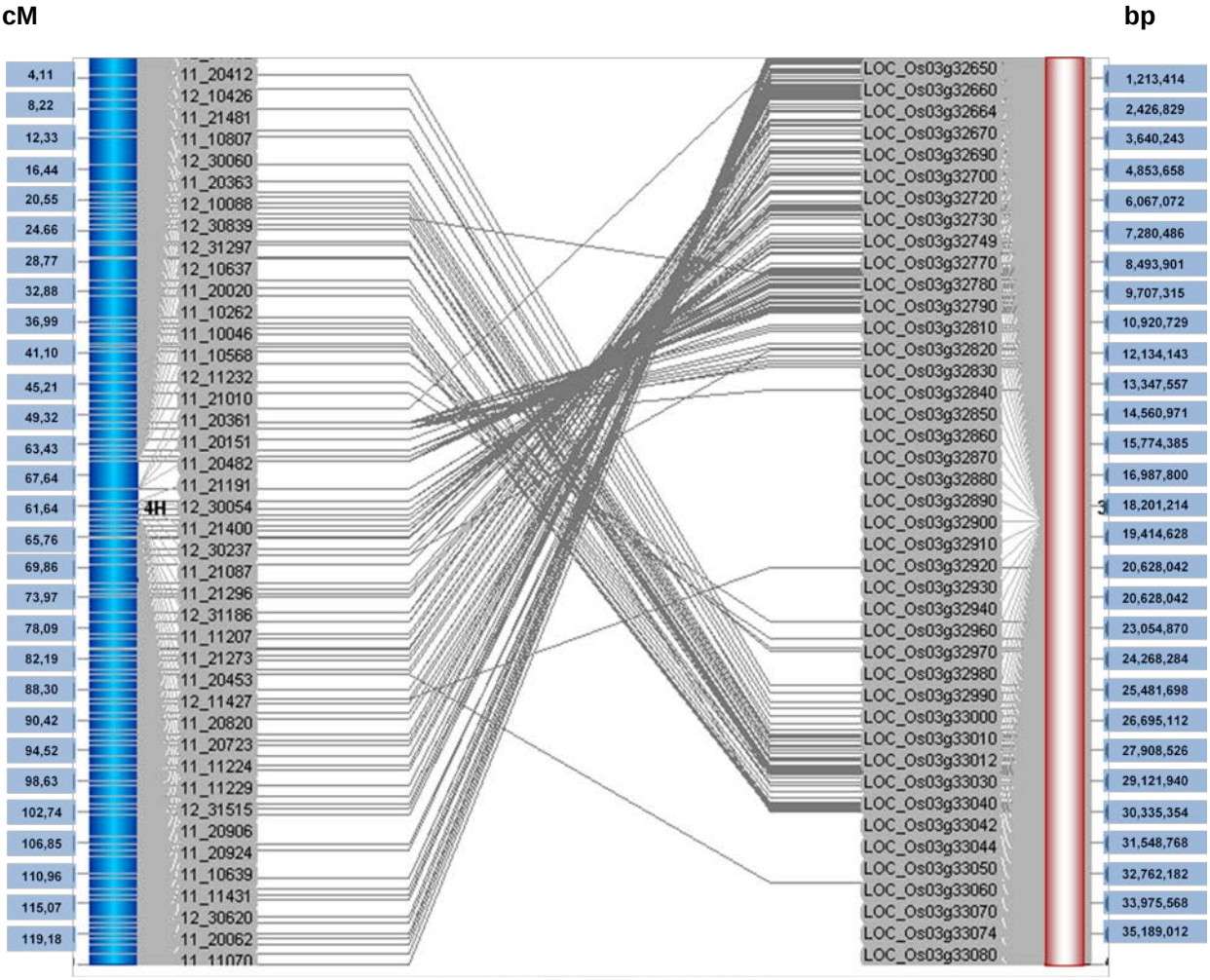
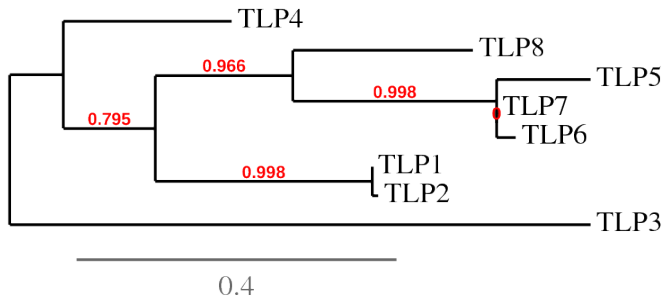


Figure 5.2: Figure a) and b) represents phylogenetic relationship showing the relatedness of the deduced full length amino acid sequence of barley HvTLP8. The Phylogenetic tree was generated using MEGA 6 program. The scale represents the frequency of amino acid substitution between sequences as determined by poisson evolutionary distance method.

a)



b)

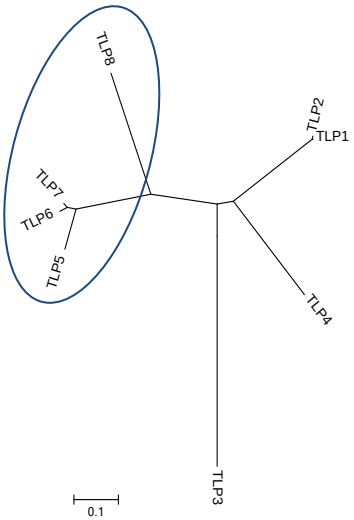
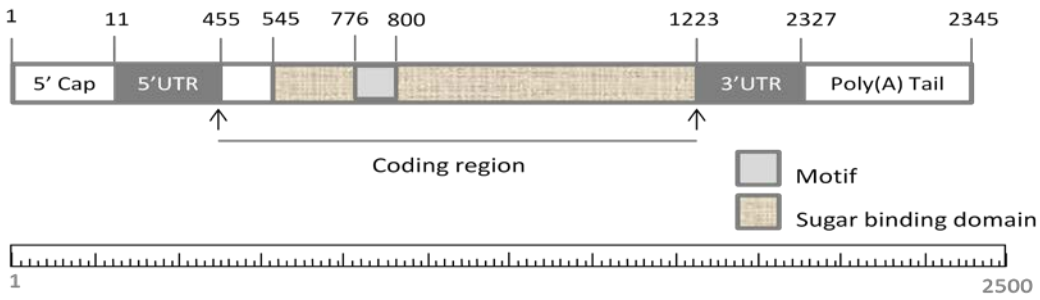


Figure 5.3: a) Structure of *HvTLP8* mature mRNA, with a scale 0 to 2500 bp. Various regions with their sequence lengths are indicated. b) Presence of an acidic pocket (red) in the three dimensional structure of HvTLP8 protein. This pocket is created at the motif site from beta-hairpins, and is believed to be involved in binding of β-glucan. c) Motif (CQTGDCGG) with possible glycosylation site.

a)



b)

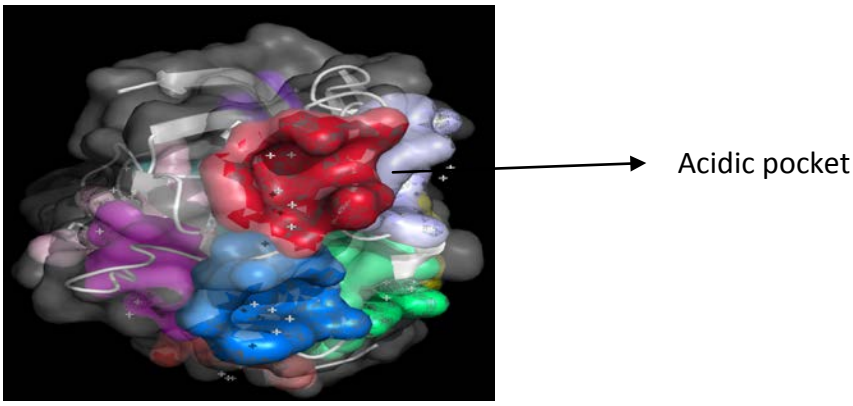
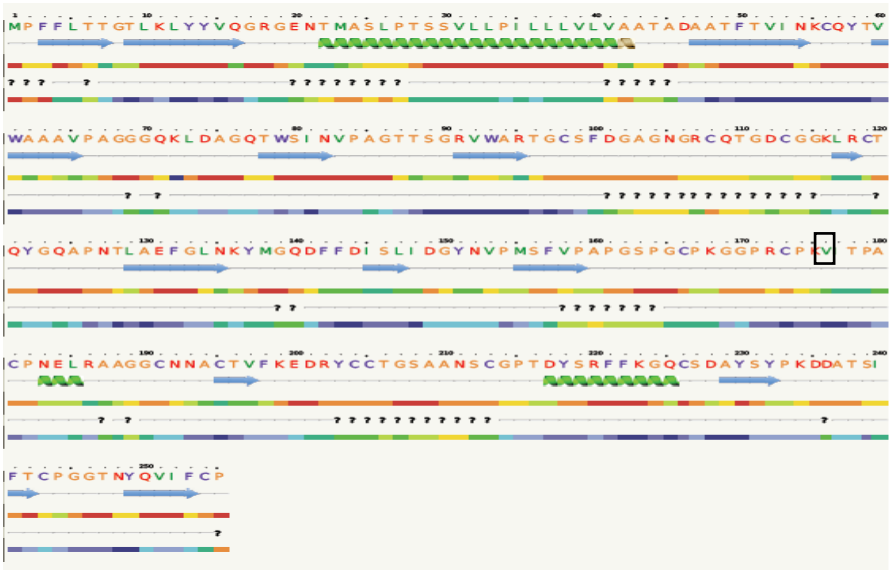


Figure 5.4: Secondary structure prediction of HvTLP8 in; a) Morex (malting variety) and b) Bowman (feed variety), based on data obtained from barley whole genome sequencing project. Amino acid sequence showing structural modifications are indicated with a key. c) Dark black outlined box indicate the presence of different amino acid (V) in Morex and (G) in Bowman varieties. Protein comparison shows a normal TLP signature in morex (green) and altered (yellow) in bowman barley variety.

a)



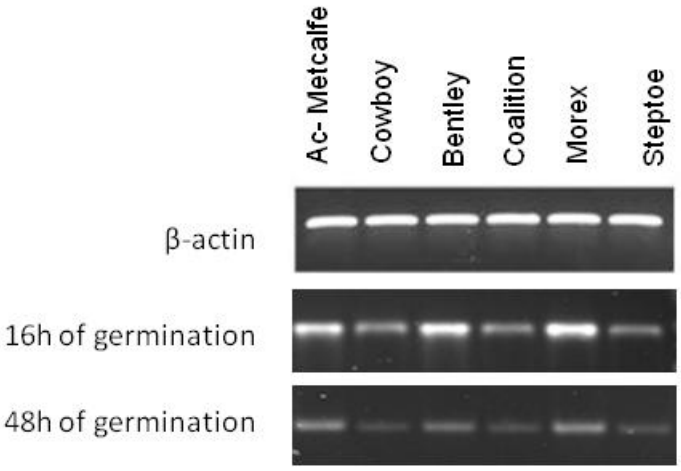
b)

c)

```
Morex  MPFFLTTGTLKLYYVQGRGENTMASLPTSSVLLPILLLVLVAATADAATFTVINKCQYTV 60
Bowman MPFFLTTGTLKLYYVQGRGENTMASLPTSSVLLPILLLVLVAATADAATFTVINKCQYTV 60

Morex  WAAAVPAGGGQKLDAGQTWSINVPAGTTSGRVWARTGCSFDGAGNGRCQTGDCGGKLRCT 120
Bowman WAAAVPAGGGQKLDAGQTWSINVPAGTTSGRVWARTGCSFDGAGNGRCQTGDCGGKLRCT 120

Morex  QYGQAPNTLAEFGLNKYMGQDFFDISLIDGYNVPMSFVPAPGSPGCPKGGPRCPKVITPA 180
Bowman QYGQAPNTLAEFGLNKYMGQDFFDISLIDGYNVPMSFVPAPGSPGCPKGGPRCPKGITPA 180

Morex  CPNELRAAGGCNNACTVFKEDRYCCTGSAANSCGPTDYSRFFKGQCSDAYSYPKDDATSI 240
Bowman CPNELRAAGGCNNACTVFKEDRYCCTGSAANSCGPTDYSRFFKGQCSDAYSYPKDDATSI 240

Morex  FTCPGGTNYQVIFCP 255
Bowman FTCPGGTNYQVIFCP 255
```

Figure 5.5: a) Gel representation of expression profiles of *HvTLP8* and housekeeping gene (β-actin) among malting and feed varieties using semi-quantitative RT-PCR at different stages of germination. b) Bar graph represents qRT-PCR expression profile of *HvTLP8 gene* in duplicates. Ac Metcalfe, Bentley and Morex are the malting varieties which are up-regulated whereas Cowboy, Coalition and Steptoe are feed varieties which are down-regulated during 16h of germination.

a) Semi quantitative PCR (RT-PCR)



b) Quantitative PCR (qRT-PCR) at 16h of germination

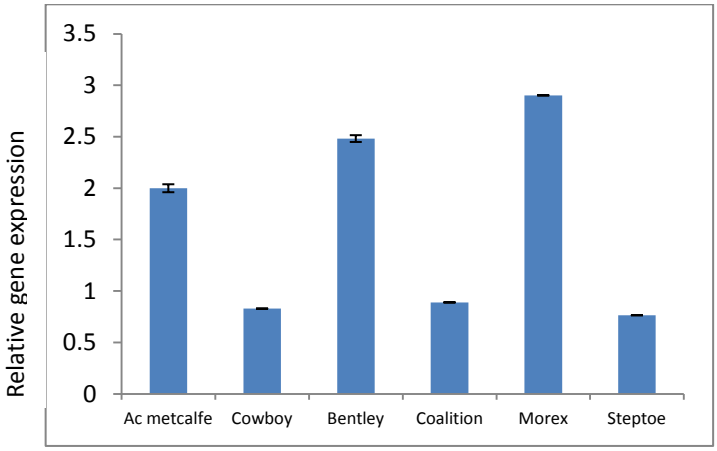


Figure 5.6: Amount of β-glucan at different stages of germination (0, 16 and 48hr).All the malting varieties has lower amount of initial β-glucan compared to feed varieties. As germination progress to 16 and 48hr, the amount of β-glucan considerably dropped in all malting varieties, whereas the decrease in feed varieties was significantly less. X-axis represent the malting (1-3) and feed (4-6) and control, whereas Y-axis represent the amount of β-glucan in percentage (w/w).

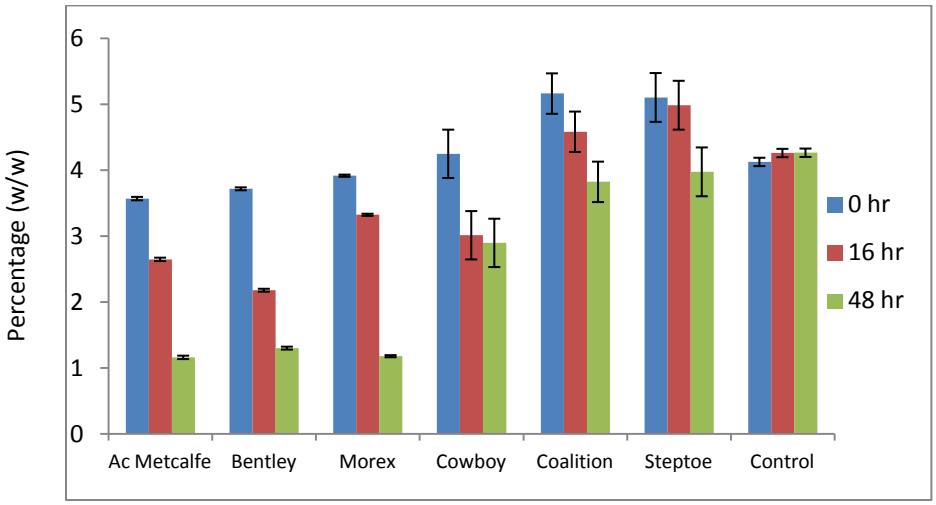


Figure 5.7: Total proteins were extracted from different varieties of barley seeds, and TLP were precipitated using ammonium acetate precipitation and resolved on SDS PAGE. a) Glycosylation of malting and non-malting varieties observed by staining with glycosylation staining kit. Malting varieties show a high degree of florescence, depicting higher β-glucan binding than non-malting. b) SDS PAGE gel showing TLP after binding with β-glucan. c) Immunoblotting of partially purified TLPs with anti-TLP antibody on a nitrocellulose membrane. A small size difference between the bound and unbound proteins in case of malting and no differences in non-malting varieties was observed. 1-6 are malting varieties and 7-12 are non-malting. Bands 1, 3, 5, 7, 9, 11 are normal proteins and 2, 4, 6, 8, 10, 12 are β-glucan bound proteins. NB represents non-bound proteins, and B represents bound proteins.

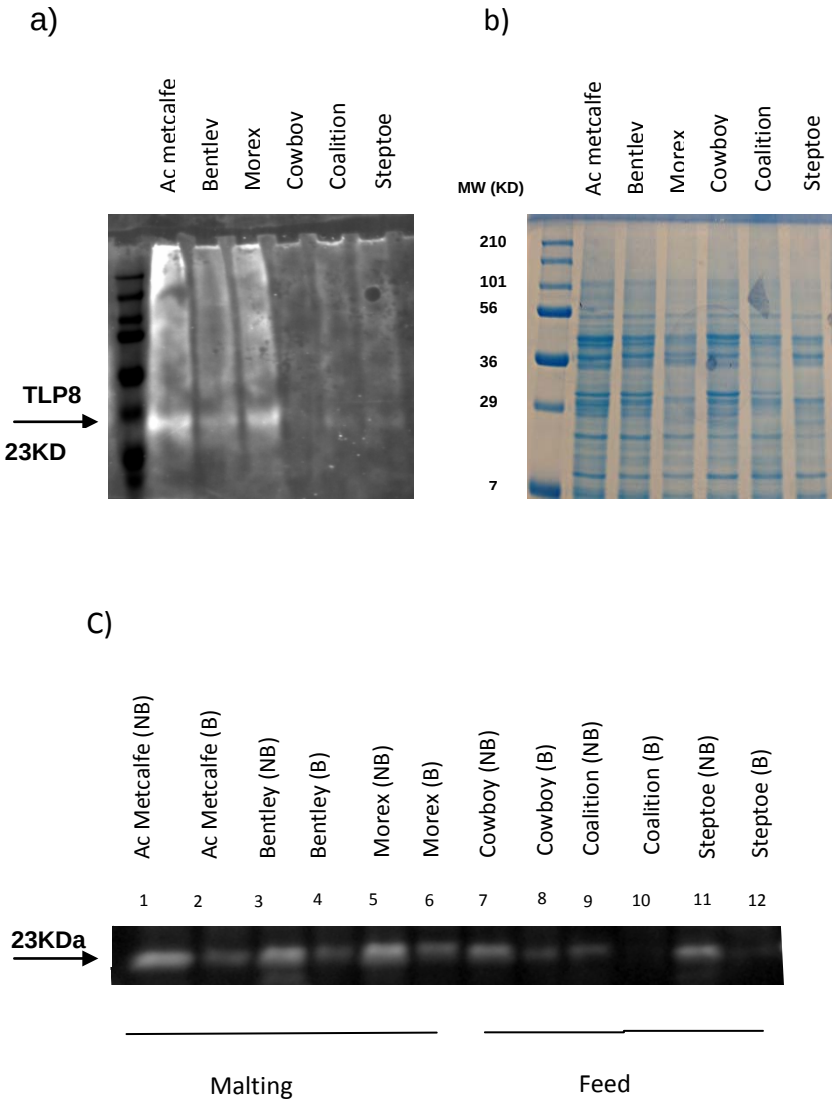
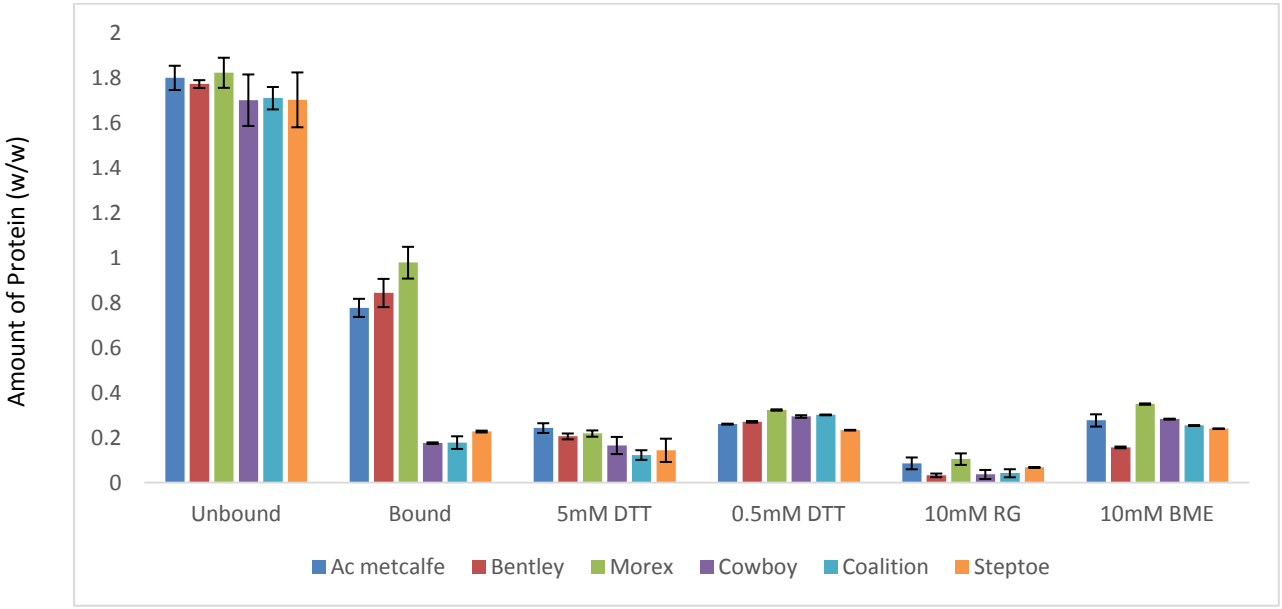


Figure 5.8: ELISA based biochemical assay designed to determine the binding ability of HvTLP with β-glucan (pachyman) and effect of different sulfhydry groups on the binding capacity of HvTLP8 and β-glucan. X axis represent Ac Metcalfe, Bentley and Morex as malting varieties and Cowboy, Coalition and Steptoe as feed varieties, with various reducing agents as treatments and Y-axis represent amount of protein. Bound proteins were calculated by subtracting the total TLPs from the unbound left in the supernatant. Group 1 represent normal unbound protein, group 2 is proteins bound to 5% β-glucan, group 3 is 5mM DTT, group 4 is 0.5mN DTT, group 5 is 10mM reduced glutathione and group 6 is 10 mM β-mercaptoethanol.



5.5 Discussion

In the present study we have employed comparative genomic and bioinformatic approaches to isolate *HvTLP8* gene from QTL2 region of barley chromosome 4H. Characterization of this gene and its protein product strongly suggest that HvTLP8 is likely to play an important role in determining the malting quality in barley. QTL2 region of barley 4H chromosome has long been known to be involved in determining malting quality. Traits like amount of malt extract (ME), wort protein, soluble/total protein (S/T), diastatic power (DP), alpha-amylase, β-glucan (BG), kernel plumpness, dormancy and free amino nitrogen (FAN), have been shown to affect malting at different levels. QTL2 is a segment of 15.8 cM in size which is located near the telomeric end of 4H chromosome has been a key target for improvement of malting quality in breeding programs using marker-assisted selection. However, given that QTL2 is a large region of the chromosome and that malting quality is multigenic trait, it has been challenging to pinpoint individual genes involved in malting trait. The present study shows the successful use of comparative genomic and bioinformatic techniques to isolate and characterize genes relevant to malting trait. Since malting involves grain germination and, therefore, hydrolytic enzyme action for degrading the storage macromolecular food reserves, it is not surprising that QTL2 harbors genes for many hydrolytic enzymes. In this respect, most of candidate genes in the QTL2 region function as starch breakdown enzymes, embryo storage proteins, stress responsive genes, transport, and cell signaling proteins.

Barley varieties for the present purpose are divided into two groups, malting varieties and feed varieties. The expression level of *HvTLP8* is consistently higher in malting varieties than in the feed varieties suggesting its possible relationship to malting quality. Phylogenetic analysis revealed relatedness of eight barley TLPs, with a conserved evolutionary relationship at the protein level. TLPs 1-4 form a single clade distinct from TLPs 5-8.This observation can be related to the presence of a negatively charged cleft caused by acidic residues in TLPs 5-8, but absent in TLPs 1-4. The HvTLP8 amino acid sequence also possesses this acidic cleft, potentially involved in β-glucan binding.

β-glucans and products of their incomplete degradation by glucanases pose several problems in the brewing process if present in excess amounts, and hinder the diffusion of enzymes, germination, reduces malt extract (ME) percentage and causes difficulty in filtration (Jonkova and Surleva 2013). Traditionally, barley varieties with lower β-glucan content are used for malting and those with higher β-glucan content are used as feed varieties. Even though final β-glucan content varies significantly during germination and malting process among malting and feed varieties but difference in initial β-glucan content prior to germination was not that huge between these varieties. This suggests that the level of β-glucan is also under the control of other factors. One might be the presence of β-glucan binding protein - HvTLP8. Previous studies report that barley TLPs has a high degree of protein-polysaccharide binding specificity with β-glucan (Trudel et al., 1998; Osmond et al., 2001).

The structure of HvTLP8 protein revealed a polysaccharide-binding motif (CQTGDCGG). Furthermore, an analysis of three dimensional folding structure of HvTLP8 showed a negatively charged acidic cleft (pocket), known to capable of binding different ligands. Such acidic cleft was also found in TLPs with known antifungal activity for binding to a specific receptor (Koiwa et al. 1999; Min et al. 2004). Higher expression of *HVTLP8* in malting varieties compared to feed, result in higher accumulation of the HvTLP8 protein in malting varieties. This suggests that *HvTLP8* is regulated at transcriptional level. Another possible reason for the differential regulation of *HvTLP8* could be the presence of a W-box promoter sequence (5’ TGAC 3’) upstream of the gene. Regulatory effects of these sequences have been previously demonstrated in rice TLPs (Hiroyuki et al., 2008).

Covalent binding of a carbohydrate moiety to protein is a common phenomenon known as glycosylation. The present study demonstrates that HvTLP8 binds to β-glucan. The majority of β-glucan binding occurred during the germination stages (16h-48h) of malting process. Following 48 hr of germination the amount of β-glucan was reduced by ~60% in malting and only by ~20% in feed varieties ((Figure 5.6). Interestingly, higher expression of *HvTLP8* was observed at 16h germination which may lead to higher accumulation of HvTLP8 protein thus greater binding to β-glucan and in turn less

amount of residual β-glucan in downstream malting process (48 h). This could be concomitant with glucanase activity of barley TLPs as indicated by its ability to digest storage glucan (Liu et al., 2010).

Quantification of such binding by ELISA showed a higher binding in malting varieties than in feed varieties. In spite of the fact that the antibody used in the assay was not specific to HvTLP8, the difference in the total binding between malt and feed varieties was evident. If the presence of excess β-glucans lowers the latter's quality, then clearly HvTLP8 has an important role improving the quality of malt. Further from ELISA results with differential effect of redox potential reactions, it indicates that HvTLP8 is a target of reducing agents like reduced glutathione, DTT and β-mercaptoethanol, which disrupts the disulphide linkages between cysteine residues and thus decreases the binding of β-glucan. Interestingly, we found that that reduced glutathione disrupts the binding of β-glucan and HvTLP8, to a large extent. This indicates that HvTLP8 might be targeted by barley thioredoxin (Trx) system. Trx system has been shown to have a key role in germinating barley seeds (Hägglund et al., 2013).

HvTLP8 falls in the PR5 (pathogenesis related) class of proteins (Christensen et al. 2002), possessing antifungal activity, and there is no previous report of HvTLP8 playing a role in malting quality. Although HvTLP8 is not directly related to previousely known malting related enzymes, our study indicates that it is playing a crucial role in controlling the amount of β-glucan during malting process via redox-regulated glycosylation.

5.6 Acknowledgement

This work was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada, Brewing and Malting Barley Research Institute and the NSERC Collaborative Research and Development grants No NSERC RGPIN 371804-10 and CRDPJ 385559 – 09 to Jaswinder Singh. The authors are thankful to Prof. Raj Dhindsa, McGill University for critically reading the manuscript.

.

Chapter VI

General conclusions and future studies

Malting quality of barley is an economically important phenotype and represents a complex, multi-component trait. Genetic improvement of malting quality is impaired by its quantitative nature and low heritability. Efforts have been undertaken to genetically dissect this trait and to localize individual quantitative trait loci (QTL) on the genetic map of barley. One major QTL complex, QTL2, mapped on the short arm of chromosome 4H, affects several malting quality parameters. The studies presented in this thesis represent an important contribution to understanding the role of QTL2 in malting quality traits, using transposon and comparative genomic approaches. We generated a set of 22 *Ds* mutants using transposon insertions and identified 24 gene candidates using gene synteny between barley and rice chromosomes to dissect QTL2. Chapter III to V, of this thesis report the detection and characterization of malting quality genes in barley. We hope that this knowledge will lead to better understanding of the malting quality traits and their effect on beer quality.

In chapter III, we initiated a transposon-based functional genomics approach to dissect the QTL2 region. It was hypothesized that the *Ac/Ds* transposon system is an effective tool for dissection of QTL regions, and the major objective was to saturate the QTL-2 region by reactivation of *Ds* transposons from the TNP-29 line using conventional and tissue culture approaches. In the conventional approach, the parent line TNP-29 was reactivated by crossing with 25B (*Ac*TPase expressing line) to activate the *Ds* transposon. In the tissue culture (In-vitro) approach AcTPase was transiently expressed to activate the *Ds* transposon in candidate parent lines. We observed a 3-fold increase in the reactivation frequency when in-vitro approach was used. Identification of unique flanking sequences, using HE-TAIL PCR and iPCR from these populations, reveled the new location of *Ds* in the barley genome and provided new transposon mutants especially in malting related genes such as *β-GAL1* and a *β-amylase*-like gene. We identified flanking sequence information of ten new *Ds* insertions. This study resulted in

the development of a new source population to facilitate the cloning of genes in important malting-quality QTL regions. The results from this study were published in Functional and Integrative Genomics in 2012.

Chapter IV highlighted the location of flanking *Ds* sequences on the barley linkage map. We earlier generated a large number of *Ds* lines by crossing TNP-29 with an *Ac*TPase expressing line and F2 progeny were screened for *Ds* insertions at new locations. Further characterization of these *Ds* mutants, required their location on the barley map. In this study, we mapped all the *Ds* insertions located on different chromosomes and found that 19 % were in the QTL2 region. Mapping was carried out using a sequence based approach, and a linkage map was generated updating the current barley map. We also confirmed the map position using the barley sequence database available (IBGSC 2012). With the ongoing effort to develop new *Ds* insertions, 12 additional flanking sequences were generated apart from Singh et al., 2012 (chapter III), using an adapter ligation method. All 22 flanking sequences were located on the barley map, with an individual linkage map constructed for each chromosome. Part of this study was published in a collaborative publication (Brown et al., 2015). Locating the *Ds* loci on the barley map was a step to obtain a better understanding of the QTL2 malting region and to study the candidate malting genes in this region.

In chapter V, we functionally characterized malting candidate genes identified in transposon and synteny-based study. In this study, the QTL2 region was further dissected using a comparative based syntenic study with rice. We identified 24 candidates, from rice that were syntenic to QTL2 region, and which have a possible roles in malting. With the synteny approach most of the candidates obtained corresponded to ESTs expressed in germination and malting stages. One candidate gene, the barley thaumatin like protein (*HvTLP8*) showed differential expression among commonly used malting and feed barley varieties. HvTLP8 was highly expressed in malting varieties compared to feed varieties. Further, we inferred that HvTLP8 binds β-glucan during germination stages of malting, and helps to sequester it. Excess β-glucan causes problems in the beer lautering and filtration process. The most desirable malting barleys are those that can combine a rapid rate of β-glucan degradation early in malting

with relatively low initial β-glucan level (Henry, 1988). Our transcriptomic and biochemical assays suggest our hypothesis and project major and unique role of HvTLP8 for superior malting quality.

Conclusively, new *Ds* insertion lines can be generated through the reactivation of the *Ds* transposon using the *Ac* transposase. In addition, further improvements of these methods, as described in chapter 4 and 5, can be utilized to increase the efficiency of generating *Ds* insertion lines. The generation of new *Ds* insertion lines will contribute to the effort of saturation mutagenesis of the QTL2.

Future studies

Expression profile of putative candidate malting genes.

We identified a number of candidate genes using transposon-based mutagenesis and synteny-based comparative approaches. These candidates may have a roles in controlling malting traits. Candidates like *β Amylase like gene, β GAL1 gene, and Glucan endo-1,3-beta-glucosidase* are putative malting genes that can be studied further. Functional characterization of these genes will open up new avenues in malting and brewing industries.

Micromalting studies of candidate lines with Ds insertions in the malting quality QTLs.

This study can be done on individual *Ds* insertion lines to determine the effect of these genes on malting quality. In addition, micromalting studies can be done for lines developed thought gene pyramiding of *Ds* insertion lines with malting quality determining characteristics. This study will be able to elucidate the genetics and environmental effects of these genes on malting quality of barley, Successful identification of these genes will facilitate the development of breeding programs for better malting varieties.

Gene knockout and *silencing studies.*

We have shown the involvement of *HvTLP8* gene in controlling the amount of β-glucan available in the malting process. Generating transposon mutant line for *HvTLP8*, by further saturating the QTL2 region using *Ds* reactivation, and performing gene

silencing studies using RNA interference, will facilitate the studies to characterize its functions.

Expression of HvTLP8 gene in E. coli.

HvTLP8 has an important role in controlling malting traits. Addition of purified protein to non-malting varieties has the potential to improve their malting characteristics. Commercial production of this protein in an expression system like *E.coli* can enhance its production and hence improve the quality of beer production.

Literature Cited

Abrouk M, Murat F et al. (2010) Palaeogenomics of plants: synteny-based modelling of extinct ancestors. Trends Plant Sci **15**:479–487.

Adamic E B. Barley and malting. In: Broderick, H. M. (Ed), *The Practical Brewer*. Madison, WI: Master Brewers Association of the Americas, pp. 21–39 (1977).

Alonso J M and Ecker J R (2006). "Moving forward in reverse: genetic technologies to enable genome-wide phenomic screens in Arabidopsis." Nat Rev Genet **7**(7): 524-536.

Anand A, Zhou T et al. (2003). "Greenhouse and field testing of transgenic wheat plants stably expressing genes for thaumatin-like protein, chitinase and glucanase against Fusarium graminearum." Journal of Experimental Botany **54**(384): 1101-1111.

Antre A H and Bhat R S (2013). "An Ac/Ds-mediated Insertional Mutagenesis System for Functional Genomics in Sorghum." Molecular Plant Breeding **4**.

Ariyadasa R, Mascher M et al. (2014). "A Sequence-Ready Physical Map of Barley Anchored Genetically by Two Million Single-Nucleotide Polymorphisms." Plant Physiology **164**(1): 412-423.

Arumuganathan K and Earle E (1991). "Nuclear DNA content of some important plant species." Plant Molecular Biology Reporter **9**(3): 208-218.

Ashburner M, Ball C A, et al. (2000) Gene Ontology: tool for the unification of biology. Nature genetics 25: 25-29.

Ayliffe M A and Pryor A J (2009). "Transposon-based activation tagging in cereals." Functional Plant Biology **36**(10-11): 915-921.

Ayliffe M A Pallotta M, et al. (2007). "A barley activation tagging system." Plant Molecular Biology **64**(3): 329-347.

Badr A K M, Sch R et al. (2000). "On the Origin and Domestication History of Barley (*Hordeum vulgare*)." Mol Biol Evol **17**(4): 499-510.

Baker B, Schell J, et al. (1986). "Transposition of the maize controlling element Activator in tobacco." Proceedings of the National Academy of Sciences of the United States of America **83**(13): 4844-4848.

Balyan H S, Sreenivasulu N et al. (2008). Mutagenesis and High-Throughput Functional Genomics in Cereal Crops: Current Status. Advances in Agronomy. L. S. Donald, Academic Press. **Volume 98:** 357-414.

Bamforth C. (1982). "Barley β-glucans. Their role in malting and brewing." Brew. Dig **57**(6): 22-27.

Bamforth C W, and Barclay A H (1993). Malting technology and the uses of malt. p. 297-354. In A.W. MacGregor and R.S. Bhatty (ed.) Barley Chemistry and Technology. Am. Assoc. Cereal Chemists, St. Paul, MN.

Bartoš J, Paux E, et al. (2008). "A first survey of the rye (Secale cereale) genome composition through BAC end sequencing of the short arm of chromosome 1R." BMC Plant Biology **8**(1): 95.

Batalia M A, Monzingo A F, et al. (1996). "The crystal structure of the antifungal protein zeamatin, a member of the thaumatin-like, PR-5 protein family." Nat Struct Mol Biol 3(1): 19-22.

Baum M, Grando S, et al. (2003). "QTLs for agronomic traits in the Mediterranean environment identified in recombinant inbred lines of the cross 'Arta' x H-spontaneum 41-1." Theoretical and Applied Genetics **107**(7): 1215-1225.

Beecher B, Beecher, et al. (2002). "Expression of wild-type pinB sequence in transgenic wheat complements a hard phenotype." TAG Theoretical and Applied Genetics **105**(6): 870-877.

Bennetzen J L, Ma J, Devos KM (2005) Mechanisms of recent genome size variation in

Bhatty, R.S. (1992). Beta-glucan content and viscosities of barleys and their roller-milled flour and barn products. Cereal Chemistry ***69***, 469-471.

Bonierbale M W, Plaisted R L et al. (1988). "RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato." Genetics **120**(4): 1095-1103.

Brandazza A, Angeli S, et al. (2004). "Plant stress proteins of the thaumatin-like family discovered in animals." Febs Letters 572(1): 3-7.

Brettell R I S, Dennis E S (1991) Reactivation of a silent Ac following tissue culture is associated with heritable alterations in its methylation pattern. Mol Gen Genet 229:365–372.

Brown R H, Singh J, et al. (2015) Behavior of a modified Dissociation element in barley: A novel tool for genetic studies and for breeding transgenic barley. Molecular Breeding 35: 85 (DOI 10.1007/s11032-015-0193-9).

Brown R H, Dahleen L, et al. (2012). "An Efficient Method for Flanking Sequence Isolation in Barley." Crop Science 52(3): 1229-1234.

Brown R H, Dahleen L, et al. (2014). "Registration of the Barley Transposon-Tagged Population I: Seventy Lines Each with a Single, Unique Site of Ds Insertion." J. Plant Reg. **8**(2): 226-230.

Bruce, P M and Robert A M (2003). "Transposon Mutagenesis in the Study of Plant Development." Critical Reviews in Plant Sciences 22: 1-35.

Burge C, Karlin S (1997) Prediction of complete gene structures in human genomic DNA. Journal of molecular biology 268: 78-94.

Büschges R, Hollricher K, et al. (1997). "The Barley Mlo Gene: A Novel Control Element of Plant Pathogen Resistance." Cell **88**(5): 695-705.

Caldwell D G (2004). "A structured mutant population for forward and reverse genetics in Barley (Hordeum vulgare L.)." The Plant Journal **40**(1): 143.

Chin H, Choe M, et al. (1999). "Molecular analysis of rice plants harbouring an Ac/Ds transposable element-mediated gene trapping system." Plant J **19**: 615 - 623.

Christensen A B, Cho B H O, et al. (2002). "The molecular characterization of two barley proteins establishes the novel PR-17 family of pathogenesis-related proteins." Molecular plant pathology 3(3): 135-144.

Chun J U, Griffith M (1998) Variation of the antifreeze proteins during cold acclimation among winter cereals and their relationship with freezing resistance Korean J. Crop Sci., 43 (1998), pp. 172–178

Chul et al. (2002) Reprogramming of the activity of activator/dissociation transposon family during plant regeneration in rice. Mol Cells 14:231–237

Chutimanitsakun Y, Nipper R, et al. (2011). "Construction and application for QTL analysis of a Restriction Site Associated DNA (RAD) linkage map in barley." BMC Genomics **12**(1): 4.

Close T J, Bhat P R, et al. (2009). "Development and implementation of high-throughput SNP genotyping in barley." Bmc Genomics **10**: 13.

Close T, Wanamaker S, et al. (2007) HarvEST. In: Edwards D, editor. Plant Bioinformatics: Humana Press. pp. 161-177.

Close T J, Wanamaker S I, et al. (2004). "A New Resource for Cereal Genomics: 22K Barley GeneChip Comes of Age." Plant Physiol. **134**(3): 960-968.

Colasanti J, Yuan Z, Sundaresan V (1998) The indeterminate gene encodes a zinc finger protein and regulates a leaf-generated signal required for the transition to flowering in maize. Cell 93:593–603

Collard B M, Jahufer, et al. (2005). "An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts." Euphytica **142**: 169 - 196.

Collins H M, Panozzo J F, (2003) Mapping and validation of chromosome regions associated with high malt extract in barley (Hordeum vulgare L.). Australian Journal of Agricultural Research 54: 1223-1240.

Consortium, I. B. G. S. (2012). "A physical, genetic and functional sequence assembly of the barley genome." Nature 491(7426): 711-716.

Cooper L D, Marquez-Cedillo L, et al. (2004). "Mapping *Ds* insertions in barley using a sequence-based approach." Molecular Genetics and Genomics **272**(2): 181-193.

Coupland G, Baker B, et al. (1988) Characterization of the maize transposable element-Ac by internal deletions.EMBO J 7:3653–3659

Cowperthwaite M, Park, W, et al. (2002). Use of the transposon *Ac* as a gene-searching engine in the maize genome. Plant Cell 14: 713–726.

DeLong A, Calderonurrea A, et al. (1993) Sex determination gene tasselseed2 of maize encodes a short-chain alcohol dehydrogenase required for stage-specific floral organ abortion. Cell 74:757–768

Devos K M, Atkinson M D, et al. (1992). "RFLP-based genetic map of the homoeologous group 3 chromosomes of wheat and rye." TAG Theoretical and Applied Genetics **83**(8): 931-939.

Douchkov D. (2005). "A high-throughput gene-silencing system for the functional assessment of defense-related genes in barley epidermal cells." Molecular plant-microbe interactions **18**(8): 755.

Drader T, Johnson K et al. (2009). "Genetic and physical mapping of a high recombination region on chromosome 7H(1) in barley." Theoretical and Applied Genetics **118**(4): 811-820.

Emebiri L C (2009). "EST-SSR markers derived from an elite barley cultivar (Hordeum vulgare L. 'Morex'): polymorphism and genetic marker potential." Genome **52**(8): 665-676.

Fedoroff N V (1989) Maize Transposable elements.In: mobile DNA (eds: D.E Berg and M.M Howe), pp.375-911.American society for Microbiology, Washington. DC.

Fernandes J, Dong Q F et al. (2004). "Genome-wide mutagenesis of Zea mays L. using RescueMu transposons." Genome Biology **5**(10).

Fernández, M. Fernández, et al. (2002). "The use of ISSR and RAPD markers for detecting DNA polymorphism, genotype identification and genetic diversity among barley cultivars with known origin." TAG Theoretical and Applied Genetics **104**(5): 845-851.

Feuillet C, Travella S, et al. (2003). "Map-Based Isolation of the Leaf Rust Disease Resistance Gene Lr10 from the Hexaploid Wheat (Triticum aestivum L.) Genome." Proceedings of the National Academy of Sciences of the United States of America **100**(25): 15253-15258.

Fierens E, Rombouts S, et al. (2007). "TLXI, a novel type of xylanase inhibitor from wheat (Triticum aestivum) belonging to the thaumatin family." Biochem. J 403: 583-591.

Fierens E, et al. (2009). "Biochemical and structural characterization of TLXI, the Triticum aestivum L. thaumatin-like xylanase inhibitor." Journal of Enzyme Inhibition and Medicinal Chemistry 24(3): 646-654.

Fierens E, Gebruers K et al. (2009). "Biochemical and structural characterization of TLXI, the *Triticum aestivum* L. thaumatin-like xylanase inhibitor." Journal of Enzyme Inhibition and Medicinal Chemistry 24(3): 646-654.

Fils-Lycaon B R, et al. (1996). "A cherry protein and its gene, abundantly expressed in ripening fruit, have been identified as thaumatin-like." Plant Physiology 111(1): 269-273.

Fils-Lycaon B R, Wiersma P A, et al. (1996). "A cherry protein and its gene, abundantly expressed in ripening fruit, have been identified as thaumatin-like." Plant Physiology 111(1): 269-273.

Finnie C and Svensson B (2009). "Barley seed proteomics from spots to structures." Journal of Proteomics **72**(3): 315-324.

Fleming M and Kawakami K (1977). "Studies of the fine structure of β-d-glucans of barleys extracted at different temperatures." Carbohydrate Research **57**(0): 15-23.

Forster B P, Thompson D M, et al. (1991) Water-soluble proteins of mature barley endosperm: genetic control, polymorphism, and linkage with -amylase and spring/winter habit. Theor Appl Genet **81**: 787-792.

Fox G P, Panozzo J F, et al. (2003)."Molecular basis of barley quality." Australian Journal of Agricultural Research **54**(11-12): 1081-1101.

Ganal M W, Altmann T, et al. (2009). "SNP identification in crop plants." Current Opinion in Plant Biology **12**(2): 211-217.

Gao W, Clancy J A, et al. (2004). "Fine mapping of a malting-quality QTL complex near the chromosome 4H S telomere in barley." Theoretical and Applied Genetics **109**(4): 750-760.

Giese H, Holm-Jensen A G, et al. (1994). “Distribution of RAPD markers on a linkage map of barley”. Hereditas **120**: 267–273.

Gill B S, Appels R, et al. (2004). "A Workshop Report on Wheat Genome Sequencing: International Genome Research on Wheat Consortium." Genetics **168**(2): 1087-1096.

Gjertsen P,. β-Glucans in malting and brewing. I. Influence of β-glucans on the filtration of strong beers. Proc. Am. Soc. Brew. Chem., 1966, 113–120.

Goodrich J, Puangsomlee P, et al. (1997). "A Polycomb-group gene regulates homeotic gene expression in Arabidopsis." Nature **386**(6620): 44-51.

Graner A, Jahoor A, et al. (1991). "Construction of an RFLP map of barley." TAG Theoretical and Applied Genetics **83**(2): 250-256.

Greco R, Ouwerkerk P, et al. (2003). "Transpositional behaviour of an Ac/Ds system for reverse genetics in rice." Theoretical and Applied Genetics **108**(1): 10-24.

Grenier J, Potvin C, et al. (1999). "Some thaumatin-like proteins hydrolyse polymeric β-1, 3-glucans". The Plant Journal 19(4): 473-480.

Griffith M, Lumb C, et al. (2005) Antifreeze Proteins Modify the Freezing Process In Planta. Plant Physiology 138: 330-340.

Gupta P K, et al. (2002) Genetic mapping of 66 new microsatellite(SSR) loci in bread wheat. Theor Appl Genet 105:413–422.

Gurel S, Gurel E, et al. (2009) Efficient reproducible Agrobacterium-mediated transformation of sorghum using heat treatment of immature embryos. Plant Cell Rep 28:429–444

Hall T A (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In Nucleic acids symposium series (Vol. 41, pp. 95-98).

Hake S, Vollbrecht E, et al. (1989). Cloning Knotted, the dominant morphological mutant in maize using Ds2 as a transposon tag. EMBO J. 8, 15-22.

Han F et al. (1998). "Synteny with rice: analysis of barley malting quality QTLs and rpg4 chromosome regions." Genome **41**(3): 373-380.

Hayes P M, Jones B L (2000) Malting quality from a QTL perspective. In: Eighth International Barley Genetics Symposium, Adelaide Convention Centre, Adelaide, South Australia, pp 99–105.

Hayes P M, Liu B H, et al. (1993)."Quantitative trait locus effects and environmental interaction in a sample of North-American barley germplasm." Theoretical and Applied Genetics **87**(3): 392-401.

Hearnden P R, (2007). "A genetic map of 1,000 SSR and DArT markers in a wide barley cross." Theoretical and Applied Genetics **115**(3): 383.

Hejgaard J, Jacobsen S, et al. (1991). "2 Antifungal thaumatin-like proteins from barley-grain." Febs Letters **291**(1): 127-131.

Henry R J, (1988). "Changes in β-glucan and other carbohydrate components of barley during malting." Journal of the Science of Food and Agriculture 42(4): 333-341.

Hiroyuki K, and Terauchi R, (2008). "Regulation of expression of rice thaumatin-like protein: inducibility by elicitor requires promoter W-box elements." Plant cell reports 27(9): 1521-1528.

Hoffman, D Hoffman, et al. (2002). "Markers polymorphic among malting barley (Hordeum vulgare L.) cultivars of a narrow gene pool associated with key QTLs." TAG Theoretical and Applied Genetics **105**(4): 544-554.

Hoffman D L, and Bregitzer P (1996). Identification of reproducible PCR–RAPD markers that enable the differentiation of closely-related six-rowed malting barley (Hordeum vulgare L.) cultivars. J. Am. Soc. Brew. Chem. 54: 172–176.

Holzberg S, Brosio P, et al. (2002). "Barley stripe mosaic virus-induced gene silencing in a monocot plant." The Plant Journal **30**(3): 315-327.

Hwang Y S, and Lorenz K, (1986). "Quality of malting barleys grown at different locations in the United States and Canada." Journal of the American Society of Brewing Chemists **44**(1): 6-12.

Islamovic E, Obert D E, et al. (2014). "Quantitative trait loci of barley malting quality trait components in the Stellar/01Ab8219 mapping population." Molecular Breeding **34**(1): 59-73.

Ito Y, Eiguchi M, et al. (2004). "Establishment of an enhancer trap system with Ds and GUS for functional genomics in rice." Molecular Genetics and Genomics **271**(6): 639-650.

Izawa T, Miyazaki C, et al. (1991). "Introduction and transposition of the maize transposable element Ac in rice (Oryza sativa L.)." Molecular and General Genetics MGG **227**(3): 391-396.

Izydorczyk M, and J Dexter, (2008). "Barley β-glucans and arabinoxylans: molecular structure, physicochemical properties, and uses in food products–a review." Food Research International **41**(9): 850-868.

Jaccoud D, Peng K, et al. (2001). "Diversity Arrays: a solid state technology for sequence information independent genotyping." Nucl. Acids Res. **29**(4): e25-.

Javaid A (2009). "Fine Mapping of Short Rachilla Hair Gene (srh) in Barley and an Association Study using Flanking Molecular Markers and World Germplasms." Asian journal of plant sciences **8**.

Jiang S Y, Ramachandran S, (2010) Natural and artificial mutants as valuable resources for functional genomics and molecular breeding. Int J Biol Sci 6:228–251.

Jin Y- L, Speers R A, et al. (2004). "Barley β-glucans and their degradation during malting and brewing." Technical Quarterly-Master Brewers Association of the Americas **41**(3): 231-240.

Jones C J, Edwards K J, et al. (1997). "Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories." Molecular Breeding **3**(5): 381-390.

Jones R N (2005). "McClintock's controlling elements: the full story." Cytogenetic and Genome Research **109**(1-3): 90-103.

Jonkova G, and Surleva A, (2013). "Impact of polysaccharides of malt on filterability of beer and possibilities for their reduction by enzymatic additives." Journal of the University of Chemical Technology and Metallurgy 48(3): 234-240.

Kalendar R, Flavell A J, et al. (2011). "Analysis of plant diversity with retrotransposon-based molecular markers." Heredity **106**(4): 520-530.

Kalpana K, Maruthasalam S, et al. (2006). "Engineering sheath blight resistance in elite indica rice cultivars using genes encoding defense proteins." Plant Science **170**(2): 203-215.

Kanyuka K, Druka A, et al. (2005). "Evidence that the recessive bymovirus resistance locus rym4 in barley corresponds to the eukaryotic translation initiation factor 4E gene." Molecular plant pathology **6**(4): 449-458.

Kawahara Y, de la Bastide M, et al. (2013) Improvement of the Oryza sativa Nipponbare reference genome using next generation sequence and optical map data. Rice 6: 4.

Keller B and Feuillet C (2000). "Colinearity and gene density in grass genomes." Trends in Plant Science **5**(6): 246-251.

Keller B, Feuillet C, et al. (2005). "Map-based isolation of disease resistance genes from bread wheat: cloning in a supersize genome." Genetical Research **85**(2): 93-100.

Kelley L A, Sternberg M J E (2009) Protein structure prediction on the Web: a case study using the Phyre server. Nat Protocols 4: 363-371.

Kilian A, Chen J, et al. (1997). "Towards map-based cloning of the barley stem rust resistance genes Rpg1 and rpg4 using rice as an intergenomic cloning vehicle." Plant Molecular Biology **35**(1): 187-195.

King R C, Stansfield W D (1990) A dictionary of genetics. Oxford University Press, Oxford.

Kohli A, Prynne M, et al. (2004). "Dedifferentiation-mediated changes in transposition behavior make the activator transposon an ideal tool for functional genomics in rice." Molecular Breeding **13**(2): 177-191.

Koiwa H, Kato H, et al. (1999). "Crystal structure of tobacco PR-5d protein at 1.8 Å resolution reveals a conserved acidic cleft structure in antifungal thaumatin-like proteins." Journal of Molecular Biology 286(4): 1137-1145.

Kolesnik T, Szeverenyi I, et al. (2004). "Establishing an efficient Ac/Ds tagging system in rice: large-scale analysis of *Ds* flanking sequences." Plant J **37**: 301 - 314.

Komatsuda T, Pourkheirandish M, et al. (2007). "Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene." Proceedings of the National Academy of Sciences of the United States of America **104**(4): 1424-1429.

Konieczny A, and Ausubel F M (1993). "A procedure for mapping arabidopsis mutations using codominant ecotype-specific pcr-based markers." Plant Journal 4(2): 403-410.

Kononowicz A K, Nelson D E, et al. (1992). "Regulation of the osmotin gene promoter." Plant Cell 4(5): 513-524

Koprek T, McElroy D, et al. (2000). "An efficient method for dispersing Ds elements in the barley genome as a tool for determining gene function." Plant Journal **24**(2): 253-263.

Krattinger S G (2009). "A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat." Science **323**(5919): 1360.

Kumar S and Fladung M (2003). "Somatic mobility of the maize element Ac and its utility for gene tagging in aspen." Plant Molecular Biology 51(5): 643-650.

Kunze R and Starlinger P (1989). "The putative transposase of transposable element Ac from Zea-Mays L interacts with subterminal sequences of Ac." Embo Journal **8**(11): 3177-3185.

Kuromori T, Hirayama T, et al. (2004). "A collection of 11,800 single-copy *Ds* transposon insertion lines in Arabidopsis." Plant Journal **37**(6): 897-905.

Lamb-Palmer N, Singh M, et al. (2013). "Prokaryotic Expression and Purification of Soluble Maize Ac Transposase." Molecular Biotechnology **54**(2): 685-691.

Lander E S, Green P, et al. (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics, 1, 174–181.

Lazarow K and Lutticke S (2009). "An Ac/Ds-mediated gene trap system for functional genomics in barley." Bmc Genomics 10.

Le Guilloux V, Schmidtke P et al. (2009) Fpocket: an open source platform for ligand pocket detection. BMC bioinformatics 10: 168.

Lee G-J, Wu X, et al. (2007) Soyabean. In: Kole C (ed) Genome wide and molecular breeding in plants-Oliseeds. Springer, Heidelberg, pp 1–45

Lehmensiek A, Bovill W, et al. (2009). Genetic Mapping in the Triticeae. Genetics and Genomics of the Triticeae**:** 201-235.

Li J, et al. (2011). "Expression of RNA-Interference/Antisense Transgenes by the Cognate Promoters of Target Genes Is a Better Gene-Silencing Strategy to Study Gene Functions in Rice." Plos One **6**(3).

Li Z W and Trick H N (2005). "Rapid method for high-quality RNA isolation from seed endosperm containing high levels of starch." Biotechniques 38(6): 872,874,876.

Lin K-C, Bushnell W R, et al. (1996). "Isolation and expression of a host response gene family encoding thaumatin-like proteins in incompatible oat-stem rust fungus interactions." MPMI-Molecular Plant Microbe Interactions **9**(6): 511-522.

Liu J-J, Zamani A, et al. (2010). "Expression profiling of a complex thaumatin-like protein family in western white pine." Planta **231**(3): 637-651.

Liu J-J, Sturrock R, et al. (2010). "The superfamily of thaumatin-like proteins: its origin, evolution, and expression towards biological function." Plant cell reports 29(5): 419-436.

Lüpken T, Stein N, et al. (2014). "High-resolution mapping of the barley Ryd3 locus controlling tolerance to BYDV." Molecular Breeding **33**(2): 477-488.

Ma L, Vu G, et al. (2010). "Synteny between *Brachypodium distachyon* and *Hordeum vulgare* as revealed by FISH." Chromosome Research **18**(7): 841-850.

Manly K. F, Cudmore J R H, et al. (2001). "Map Manager QTX, cross-platform software for genetic mapping." Mammalian Genome **12**(12): 930-932.

Manners, D. J. and Wilson G. (1976). "Purification of malted-barley endo-β-D-glucanases by ion-exchange chromatography: some properties of an endo-barley-β-D-glucanase." Carbohydrate Research **48**(2): 255-264.

Marsch-Martinez, N. and A. Pereira (2011). "Activation tagging with En/Spm-I /dSpm transposons in Arabidopsis." Methods in molecular biology (Clifton, N.J.) **678**: 91-105.

Mather D E, Tinker N A, et al. (1997). "Regions of the genome that affect grain and malt quality in a North American two-row Barley cross." Crop Sci **37**(2): 544-554.

Mayer K F X, Martis M, et al. (2011). "Unlocking the Barley genome by chromosomal and comparative genomics." Plant Cell **23**(4): 1249-1263.

McClear B V and Glennie-Holmes M (1985). "Enzymic quantification of (1→ 3)(1→ 4)-β-D-GLUCAN in barley and malt." Journal of the Institute of Brewing 91(5): 285-295.

McElroy D, Louwerse J, et al. (1997). "Development of a simple transient assay for *Ac/Ds* activity in cells of intact barley tissue." Plant J **11**: 157 - 165.

Mester D I, Ronin Y I, et al. (2003). "Efficient multipoint mapping: making use of dominant repulsion-phase markers." TAG Theoretical and Applied Genetics **107**(6): 1102-1112.

Min K, Ha S C et al. (2004). "Crystal structure of osmotin, a plant antifungal protein." Proteins: Structure, Function, and Bioinformatics 54(1): 170-173.

Molina-Cano, J. L. (1987). "The EEC Barley and Malt Committee Index for the Evaluation of Malting Quality in Barley and its Use in Breeding." Plant Breeding 98(3): 249-256.

Nakagawa Y, Machida C, Machida Y, Toriyama K (2000) Frequency and pattern of transposition of the maize transposable element Ds in transgenic rice plants. Plant Cell Physiol 41:733–742

Nandha P S and Singh J (2014). "Comparative assessment of genetic diversity between wild and cultivated barley using gSSR and EST-SSR markers." Plant Breeding **133**(1): 28-35.

Nelson J C, Sorrells M E, et al. (1995). "Molecular Mapping of Wheat: Major Genes and Rearrangements in Homoeologous Groups 4, 5, and 7." Genetics **141**(2): 721-731.

Nilan, R.A. 1964. The cytology and genetics of barley. Washington State University Press, Pullman, WA

Osmond, R. I., et al. (2001). "Binding interactions between barley thaumatin-like proteins and (1, 3)-β-D-glucans." European Journal of Biochemistry **268**(15): 4190-4199.

Parinov S, Sevugan M, et al. (1999). "Analysis of Flanking Sequences from Dissociation Insertion Lines: A Database for Reverse Genetics in Arabidopsis." Plant Cell **11**(12): 2263-2270.

Pastori G M, Huttly A et al. (2007). "The maize Activator/Dissociation system is functional in hexaploid wheat through successive generations." Functional Plant Biology **34**(9): 835-843.

Paux E and Sourdille P (2009). A Toolbox for Triticeae Genomics. Genetics and Genomics of the Triticeae**:** 255-283.

Perry D J, Fernando U, et al. (2013). "Simple sequence repeat-based identification of Canadian malting barley varieties." Canadian Journal of Plant Science **94**(3): 485-496.

Peters J L, Cnudde F, Gerats T (2003) Forward genetics and map based cloning approaches. Trends Plant Sci 8:484–491

Poland J A et al. (2012). "Development of High-Density Genetic Maps for Barley and Wheat Using a Novel Two-Enzyme Genotyping-by-Sequencing Approach." Plos One **7**(2).

Potokina E, et al. (2004). "Functional association between malting quality trait components and cDNA array based expression patterns in barley (Hordeum vulgare L.)." Molecular Breeding **14**(2): 153-170.

Potokina E, Prasad M, et al. (2006). "Expression genetics and haplotype analysis reveal cis regulation of serine carboxypeptidase I (Cxp1), a candidate gene for malting quality in barley (Hordeum vulgare L.)." Funct Integr Genomics **6**(1): 25-35.

Pourkheirandish M and Komatsuda T (2007). "The Importance of Barley Genetics and Domestication in a Global Perspective." Ann Bot **100**(5): 999-1008.

Qu S, Desai A, et al. (2008). "A versatile transposon-based activation tag vector system for functional genomics in cereals and other monocot plants." Plant Physiology **146**(1): 189-199.

Rafalski A (2002). "Applications of single nucleotide polymorphisms in crop genetics." Current Opinion in Plant Biology 5(2): 94-100.

Ramachandran, S. and V. Sundaresan (2001). "Transposons as tools for functional genomics." Plant Physiology and Biochemistry **39**(3-4): 243-252.

Randhawa H S, Singh J, et al. (2009). "Mapping barley Ds insertions using wheat deletion lines reveals high insertion frequencies in gene-rich regions with high to moderate recombination rates." Genome **52**(6): 566-575.

Ranki H, Sopanen T, et al. (1990). "Localization of Carboxypeptidase I in Germinating Barley Grain." Plant Physiol. **93**(4): 1449-1452.

Rebmann G, et al. (1991). "Sequence of a wheat cDNA encoding a pathogen-induced thaumatin-like protein." Plant Molecular Biology **17**(2): 283-285.

Reiss E and Horstmann C (2001). " Drechslera teres–infected barley (Hordeum vulgare L.) leaves accumulate eight isoforms of thaumatin-like proteins." Physiological and molecular plant pathology **58**(4): 183-188.

Reiss E, Schlesier B, et al. (2006). "cDNA sequences, MALDI-TOF analyses, and molecular modelling of barley PR-5 proteins." Phytochemistry **67**(17): 1856-1864.

Ros F and Kunze R (2001). "Regulation of activator/dissociation transposition by replication and DNA methylation." Genetics **157**(4): 1723-1733.

Rostoks N, Mudie S, et al., 2005b Genome-wide SNP discovery and linkage analysis in barley based on genes responsive to abiotic stress. Mol. Genet. Genomics 274: 515–527.

Sato K, Close T J, et al. (2011). "Single Nucleotide Polymorphism Mapping and Alignment of Recombinant Chromosome Substitution Lines in Barley." Plant and Cell Physiology **52**(5): 728-737.

Sato K, Nankaku N, et al. (2009). "A high-density transcript linkage map of barley derived from a single population." Heredity **103**(2): 110-117.

Schmidt E D L, et al. (1997). "A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos." Development **124**(10): 2049-2062.

Schulte D, Close T J, et al. (2009). "The International Barley Sequencing Consortium - At the Threshold of Efficient Access to the Barley Genome." Plant Physiology **149**(1): 142-147.

Shimamoto K, Miyazaki C, et al. (1993). "Trans-activation and stable integration of the maize transposable element Ds cotransfected with the *Ac* transposase gene in transgenic rice plants." Molecular and General Genetics MGG **239**(3): 354-360.

Sigrist C J A, de Castro E, et al. (2013) New and continuing developments at PROSITE. Nucleic Acids Research 41: D344-D347.

Silvar C, Perovic D, et al. (2010) "Fine mapping and comparative genomics integration of two quantitative trait loci controlling resistance to powdery mildew in a Spanish barley landrace." TAG Theoretical and Applied Genetics: 1-14.

Singh J, Freeling M, et al. (2008). "A Position Effect on the Heritability of Epigenetic Silencing." Plos Genetics **4**(10).

Singh J, Zhang S, et al. (2006). "High-frequency Ds remobilization over multiple generations in barley facilitates gene tagging in large genome cereals." Plant Molecular Biology **62**(6): 937-950.

Singh M, Lewis P E et al. (2003). "Activator mutagenesis of the pink scutellum1/viviparous7 locus of maize." Plant Cell **15**(4): 874-884.

Singh, N K, et al. (1987). "Characterization of osmotin A thaumatin-like protein associated with osmotic adaptation in plant cells." Plant Physiology **85**(2): 529-536.

Singh S, Tan H-Q et al. (2012). "Mutagenesis of barley malting quality QTLs with Ds transposons." Funct Integr Genomics **12**(1): 131-141.

Smith D, Yanai Y, et al. (1996). "Characterization and mapping of Ds-GUS-T-DNA lines for targeted insertional mutagenesis." Plant Journal **10**(4): 721-732.

Smulders M J M. and. Klerk de G J, (2011). "Epigenetics in plant tissue culture." Plant Growth Regulation **63**(2): 137-146.

Springer P (2000). "Gene Traps: Tools for Plant Development and Genomics." Plant Cell **12**: 1007 - 1020.

Sreenivasulu N, Usadel B, et al. (2008). "Barley Grain Maturation and Germination: Metabolic Pathway and Regulatory Network Commonalities and Differences Highlighted by New MapMan/PageMan Profiling Tools." Plant Physiol. **146**(4): 1738-1758.

Sreenivasulu N, Usadel B et al. (2008). "Barley grain maturation and germination: Metabolic pathway and regulatory network commonalities and differences highlighted by new MapMan/PageMan profiling tools." Plant Physiology 146(4): 1738-1758.

Stam P, (1993). "Construction of integrated genetic linkage maps by means of a new computer package: Join Map." The Plant Journal **3**(5): 739-744.

Stanca M (2003) Diversity in abiotic stress tolerance. In Diversity in Barley Edited by: von Bothmer R, van Hintum T, Knuepffer H, Sato K .Amsterdam: Elsevier Science; 2003:179-199.

Stein N, Prasad M, et al. (2007). "A 1,000-loci transcript map of the barley genome: new anchoring points for integrative grass genomics." TAG Theoretical and Applied Genetics **114**(5): 823-839.

Swatson J S, Ellis R P, 2002: Genetics and breeding of malt quality attributes. In: Slafer, G. A., Molina-Cano, J. L., Savin, R., Araus, J. L., Romagosa, I. (eds.): Barley Science: Recent Advances from Molecular Biology to Agronomy of Yield and Quality, New York: Food Products Press, 85– 114. ISBN 1-56022-910-1.

Takumi S, Murai K, et al. (1999). "Trans-activation of a maize Ds transposable element in transgenic wheat plants expressing the Ac transposase gene." TAG Theoretical and Applied Genetics **98**(6): 947-953.

Talame V, Bovina R, et al. (2008). "TILLMore, a resource for the discovery of chemically induced mutants in barley." Plant Biotechnology Journal **6**(5): 477-485. The International Barley Genome Sequencing Consortium (IBSC) (2012) A physical, genetic and functional sequence assembly of the barley genome. Nature 491:711–716. doi:10.1038/ Nature 11543.

Tan H-Q, Singh J (2011) High-efficiency thermal asymmetric interlaced (HE-TAIL) PCR for amplification of Ds transposon insertion sites in barley. J Plant Mol Biol Biotechnol 2:9–14.

Triantafillidou D, Georgatsos JG (2001) Barley β-Galactosidase: structure, function, heterogeneity, and gene origin. J Protein Chem 7:551–562.

Trudel J, Grenier J, et al. (1998). "Several thaumatin-like proteins bind to β-1, 3-glucans." Plant Physiology **118**(4): 1431-1438.

Turner, A., et al. (2005). "The pseudo-response regulator Ppd-H1 provides adaptation to photoperiod in barley." Science **310**(5750): 1031-1034.

Upadhyaya N, Zhou X-R et al. (2002). "An iAc/Ds gene and enhancer trapping system for insertional mutagenesis in rice." Funcl Plant Biol **29**: 547 - 559.

Upadhyaya N, Zhu Q-H et al. (2006). "Dissociation (*Ds*) constructs, mapped Ds launch pads and a transiently-expressed transposase system suitable for localized insertional mutagenesis in rice." TAG Theoretical and Applied Genetics **112**(7): 1326-1341.

Van Loon L C and van Kammen A, (1970). "Polyacrylamide disc electrophoresis of the soluble leaf proteins from Nicotiana tabacum var. "Samsun" and "Samsun NN". II. Changes in protein constitution after infection with tobacco mosaic virus." Virology **40**(2): 190-211.

Van Os H, Stam P, et al. (2005a) RECORD: a novel method for ordering loci on a genetic linkage map. Theor. Appl. Genet. 112(1): 30–40.

Vanderwe H and Loeve K (1972). "Isolation and characterization of thaumatin i and ii, sweet-tasting proteins from thaumatococcus-daniellii benth." European Journal of Biochemistry 31(2): 221.

Varagona M J, et al. (1992). "Alternative splicing induced by insertion of retrotransposons into the maize waxy gene." Plant Cell **4**(7): 811-820.

Varshney R K (2007). "A high density barley microsatellite consensus map with 775 SSR loci." Theoretical and Applied Genetics **114**(6): 1091.

Velazhahan R, et al. (1999). "The PR-5 family: thaumatin-like proteins." Pathogenesis-related proteins in plants. CRC Press, Boca Raton: 107-129.

Vinje M A, et al. (2011). "Differential expression of two beta-amylase genes (Bmy1 and Bmy2) in developing and mature barley grain." Planta **233**(5): 1001-1010.

Vollbrecht E, Duvick J, et al. (2010). "Genome-Wide Distribution of Transposed Dissociation Elements in Maize." The Plant Cell Online **22**(6): 1667-1685.

Voorrips R E (2002). "MapChart: Software for the Graphical Presentation of Linkage Maps and QTLs." Journal of Heredity 93(1): 77-78.

Vos P, Hogers R, et al. (1995). "AFLP: a new technique for DNA fingerprinting." Nucl. Acids Res. **23**(21): 4407-4414.

Walbot V (2000). "Saturation mutagenesis using maize transposons." Current Opinion in Plant Biology **3**(2): 103-107.

Wang J, Zhang G et al. (2004). "The changes of β-glucan content and β-glucanase activity in barley before and after malting and their relationships to malt qualities." Food Chemistry **86**(2): 223-228.

Watanabe S, Hideshima R, et al. (2009). "Map-Based Cloning of the Gene Associated With the Soybean Maturity Locus E3." Genetics **182**(4): 1251-1262.

Watanabe S, Xia Z et al. (2011). "A Map-Based Cloning Strategy Employing a Residual Heterozygous Line Reveals that the GIGANTEA Gene Is Involved in Soybean Maturity and Flowering." Genetics **188**(2): 395-U260.

Watson L and Henry R J (2005). "Microarray analysis of gene expression in germinating barley embryos (Hordeum vulgare L.)." Funct Integr Genomics **5**(3): 155-162.

Waugh R, Leader D J, et al. (2006). "Harvesting the potential of induced biological diversity." Trends in Plant Science **11**(2): 71-79.

Wei K, Xue D W, et al. (2009). "Genetic mapping of quantitative trait loci associated with beta-amylase and limit dextrinase activities and beta-glucan and protein fraction contents in barley." Journal of Zhejiang University-Science B **10**(11): 839-846.

Weld R J, Bicknell R A, et al. (2002). "*Ds* transposition mediated by transient transposase expression in Heiracium aurantiacum." Plant Cell Tissue and Organ Culture **69**(1): 45-54.

White J, Pacey-Miller T et al. (2008). "Differential LongSAGE tag abundance analysis in a barley seed germination time course and validation with relative real-time RT-PCR." Plant Science **175**(6): 858-867.

Winter P and Kahl G (1995). "Molecular marker technologies for plant improvement." World Journal of Microbiology and Biotechnology **11**(4): 438-448.

Woodward J, Fincher G B, et al. (1983). "Water-soluble (1→ 3),(1→ 4)-β-D-glucans from barley (Hordeum vulgare) endosperm. II. Fine structure." Carbohydrate Polymers **3**(3): 207-225.

Yan L, Loukoianov A, et al. (2003). "Positional Cloning of the Wheat Vernalization Gene *VRN1*." Proceedings of the National Academy of Sciences of the United States of America **100**(10): 6263-6268.

Yang, W-C, Ye D, et al. (1999). "The Sporocyteless gene of Arabidopsis is required for initiation of sporogenesis and encodes a novel nuclear protein." Genes & Development **13**(16): 2108-2117.

Yun D-J, et al. (1997). "Stress proteins on the yeast cell surface determine resistance to osmotin, a plant antifungal protein." Proceedings of the National Academy of Sciences **94**(13): 7082-7087.

Zale JM, Clancy JA, Ullrich SE, Jones BL, Hayes PM (2000) North American barley genome mapping project. Summary of barley malting quality QTL mapped in various populations. Barley Genet Newslett 30:44

Zhang G, Chen J et al. (2001). "Cultivar and environmental effects on (1→ 3, 1→ 4)-β-D-glucan and protein content in malting barley." Journal of Cereal Science 34(3): 295-301.

Zhao S, Fernald R D (2005) Comprehensive algorithm for quantitative real-time polymerase chain reaction. Journal of computational biology 12: 1047-1064

Zhao T, Palotta M, et al. (2006). "Mapped Ds/T-DNA launch pads for functional genomics in barley." Plant Journal **47**(5): 811-826.

Zhu Q-H, et al. (2004). "The anther indehiscence1 Gene Encoding a Single MYB Domain Protein Is Involved in Anther Development in Rice." Plant Physiol. 135(3): 1514-1525.

Zhu Q-H, Hoque M, et al. (2003). "*Ds* tagging of branched floretless 1 (BFL1) that mediates the transition from spikelet to floret meristem in rice (Oryza sativa L)." BMC Plant Biology **3**(1): 6.

Zohary D, and Hopf M (1993). "Domestication of Plants in the Old World Second Edition edn "Oxford Science Publications, Clarendon Press.

Appendix

License for chapter III from Springer

SPRINGER LICENSE TERMS AND CONDITIONS

Aug 22, 2014

This is a License Agreement between surinder singh ("You") and Springer ("Springer") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number 3454241402476

License date Aug 22, 2014

Licensed content publisher: Springer

Licensed content publication: Functional & Integrative Genomics

Licensed content title: Mutagenesis of barley malting quality QTLs with Ds transposons

Licensed content author Surinder Singh

Licensed content date Jan 1, 2011

Volume number 12

Issue number 1

Type of Use Thesis/Dissertation

Portion Full text

Number of copies 1

Author of this Springer article

Yes and you are the sole author of the new work

Order reference number None

Title of your thesis /dissertation: Functional genomics of malting quality QTL through saturation mutagenesis with Ds insertions in barley

Expected completion date Dec 2014

Estimated size (pages) 200

Total 0.00 CAD Terms and Conditions

Introduction

The publisher for this copyrighted material is Springer Science + Business Media. By clicking "accept" in connection with completing this licensing transaction, you agree that the fol owing terms and conditions apply to this transaction (along with the Bil ing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

Limited License

With reference to your request to reprint in your thesis material on which Springer Science and Business Media control the copyright, permission is granted, free of charge, for the use indicated in your enquiry.

Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process.

This License includes use in an electronic form, provided its password protected or on the university's intranet or repository, including UMI (according to the definition at the Sherpa website: http://www.sherpa.ac.uk/romeo/). For any other electronic use, please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com).

The material can only be used for the purpose of defending your thesis limited to university-use only. If the thesis is going to be published, permission needs to be re-obtained (selecting "book/textbook" as the type of use).

Although Springer holds copyright to the material and is entitled to negotiate on rights, this license is only valid, subject to a courtesy information to the author (address is given with the article/chapter) and provided it concerns original material which does not carry references to other sources (if material in question appears with credit to another source, authorization from that source is required as well).

Permission free of charge on this occasion does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

Altering/Modifying Material: Not Permitted

You may not alter or modify the material in any manner. Abbreviations, additions, deletions and/or any other alterations shal be made only with prior written authorization of the author(s) and/or Springer Science + Business Media. (Please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

Reservation of Rights

Springer Science + Business Media reserves al rights not specifical y granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (i) these terms and conditions and (i i) CCC's Bil ing and Payment terms and conditions.

Copyright Notice:Disclaimer

You must include the fol owing copyright and permission notice in connection with any reproduction of the licensed material: "Springer and the original publisher /journal title, volume, year of

publication, page, chapter/article title, name(s) of author(s), figure number(s), original copyright notice) is given to the publication in which the material was original y published, by adding; with kind permission from Springer Science and Business Media"

Warranties: None

Example 1: Springer Science + Business Media makes no representations or warranties with respect to the licensed material.

Example 2: Springer Science + Business Media makes no representations or warranties with respect to the licensed material and adopts on its own behalf the limitations and disclaimers established by CCC on its behalf in its Bil ing and Payment terms and conditions for this licensing transaction.

Indemnity

You hereby indemnify and agree to hold harmless Springer Science + Business Media and CCC, and their respective of icers, directors, employees and agents, from and against any and al claims arising out of your use of the licensed material other than as specifical y authorized pursuant to this license.

No Transfer of License

This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without Springer Science + Business Media's written permission.

No Amendment Except in Writing

This license may not be amended except in a writing signed by both parties (or, in the case of

Springer Science + Business Media, by CCC on Springer Science + Business Media's behalf).

Objection to Contrary Terms

Springer Science + Business Media hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Bil ing and Payment terms and conditions. These terms and conditions, together with CCC's Bil ing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and Springer Science + Business Media (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Bil ing and Payment terms and conditions, these terms and conditions shal control.

Jurisdiction

Al disputes that may arise in connection with this present License, or the breach thereof, shal be settled exclusively by arbitration, to be held in The Netherlands, in accordance with Dutch law, and to be conducted under the Rules of the 'Netherlands Arbitrage Instituut' (Netherlands Institute of Arbitration).OR:

All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by arbitration, to be held in the Federal Republic of Germany, in accordance with German law.

Other terms and conditions

You will be invoiced within 48 hours of this transaction date. Y ou may pay your invoice by credit card upon receipt of the invoice for this transaction. Please follow instructions provided at that time.

To pay for this transaction now; please remit a copy of this document along with your payment. Payment should be in the form of a check or money order referencing your

account number and this invoice number RLNK501384031.

Make payments to "COPY RIGHT CLEARANCE CENTER" and send to:

Copyright Clearance Center

Dept 001

P.O. Box 843006

Boston, MA 02284-3006

Please disregard electronic and mailed copies if you remit payment in advance. Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or

+1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.