Dissecting Barley Malting Quality QTLs with Maize *Ac/Ds* Transposons

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Abstract

Malting quality of barley is a complex but important trait for the malting and brewing industries. Several malting quality QTLs have been located on the chromosome 4H of barley. However, the genes associated with these QTLs regions are unknown. The *Ac/Ds* transposon system was used to dissect these malting quality QTLs. New single-copy *Ds* insertion lines (TNPs) were generated through sequential re-activation of *Ds* transposon in the candidate parental lines – TNP-29 and -79, in which the *Ds* insertion sites were mapped in the vicinity of the malting quality QTLs on chromosome 4H. Reactivation of *Ds* was carried out by crossing these TNPs with *Ac*TPase expressing plants as well as through *in-vitro* expression of *Ac*TPase in immature barley embryos. Furthermore, a new PCR based approach – HE-TAIL PCR was devised to expedite the detection of new transposition events. This study will contribute to a better understanding of genes involved in the barley malting quality.

Résumé

La qualité du malt de l'orge est un trait complexe mais important pour les secteurs du maltage et de l'industrie brassicole. Plusieurs QTLs associés à la qualité du malt sont localisés sur le chromosome 4H de l'orge. Cependant, les gènes associés à ces QTLs sont inconnus. Par conséquent, nous avons utilisé le système de transposons *Ac/Ds* afin de caractériser ces QTLs. De nouvelles lignées comprenant une insertion unique de l'élément *Ds* (TNPs) ont donc été produites grâce à la réactivation séquentielle du transposon *Ds* chez des lignées reconnues comme ayant un élément *Ds* unique à proximité de ces QTLs. La réactivation de l'élément *Ds* a été réalisée en croisant les lignées parentales TNP-29 et TNP-79 avec une lignée exprimant l'*Ac*TPase ainsi que par l'insertion par transformation de l'*Ac*TPase chez des embryons immatures obtenus à partir de ces mêmes lignées. De plus, nous avons développé l'approche HE-TAIL PCR afin d'accélérer la détection de nouveaux événements de transposition. Par conséquent, mes travaux contribuent à améliorer notre compréhension des mécanismes impliqués dans la régulation de la qualité du malt de l'orge.

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Contribution of Authors to Manuscripts

The presentation of this thesis is manuscript-based and is prepared according to the “Guidelines Concerning Thesis Preparation” of McGill University. Chapter 3 to 5 represent three separate manuscripts. Chapter 3 and 4 are manuscripts in preparation. Chapter 5 has been submitted to the “Biotechnology Journal”. The co-author of these manuscripts is Dr. Jaswinder Singh. The corresponding address for Dr. Singh is found at the title page for each manuscript. A detailed description of the contributions of each author was mentioned in the connecting statements before each manuscript-based chapter.

I have contributed to all three chapters presented in this thesis. My contribution consists of all work, including the design and performance of the experiments, the compilation of results and data analyses, and the preparation of manuscripts. My supervisor Dr Jaswinder Singh has provided funds throughout the course of my Master’s degree. He has also contributed to the overall design of the experiments. In addition, Dr. Jaswinder Singh has supervised the completion of my thesis, including corrections and suggestions throughout the writing of my Master’s thesis.

List of Abbreviations

2,4-D	2, 4-Dichlorophenoxy acetic acid
AA	Amylase activity
AAL	Apparent attenuation limit
ABC	ATP binding cassette
Ac/Ds	*Activator-Dissociation* system
*Ac*TPase	*Activatior* transposase
AP	Alkaline phosphatate
BAC	Bacterial artificial chromosome
BAP	6-Benzylaminopurine
BG	Malt β-glucan content
BLAST	Basic local alignment search tool
bp	Basepairs
cDNA	Complementary DNA
CI	Callus-induction
CO	Co-cultivation
CSPD	3-(4-methoxyspiro{1,2–dioxetane-3,2-(5-chloro)tricycle [3.3.1.13,7]decan}-4-yl)phenylphosphate)
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
dNTP	Deoxyneucleotide triphosphate
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dor	seed dormancy
DP	Diastatic power
dpi	Days post inoculation
Ds	*Dissociation*
DsT	*Ds* transposon
dUTP	Deoxyuridine triphosphate

EDTA	Ethylene diamine tetraacetic acid
EMS	Ethyl Methyl Sulfate
EST	Expressed sequence tags
FAO	Food and Agriculture Organization
GFP	Green fluorescent protein
GP	Golden Promise
GUS	β-glucuronidase
HE-TAIL PCR	High-efficiency TAIL PCR
hpt	hygromycin
iPCR	Nested inverse PCR
KP	Kernel plumpness
LB	Lysogeny Broth
LDL	Low-density lipoprotein
MAS	Marker assisted selection
MCE	Mixed cellulose ester
MCS	Multiple cloning sites
MDR	Muti-drug resistance
ME	Malt extract yield
mRNA	Messenger RNA
MS	Murashige & Skoog
Mu	*Mutator*
nos	Nopaline synthase
O.D.	Optical density
pAct	*Actin* Prompter
PAT	phosphinothricin acetyl transferase
PCR	Polymerase chain reaction
PES	Polyethersulfone
POT	Proton-dependent Oligopeptide Transporter
PTGS	Post-transcirptional gene silencing
pUbi	Ubiquitin promoter
QTL	Quantitative trait loci
R	Rooting
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid

RNase	Ribonuclease
RT	Rooting-transition
SAGE	Serial analysis of gene expression
SDS	Sodium dodecyl sulfate
Spm/dSpm	*Suppressor-mutator*
SSC	saline sodium citrate
T_3	3rd generation transgenic plant
TAIL PCR	Thermal asymmetric interlaced PCR
TE	Tris-EDTA
TILLING	Targeted induced local lesions in genomes
TIR	Terminal inverted repeats
TNPs	Single *Ds* insertion lines
Tris	Tris-hydroxymethyl aminomethane
Uid-A	β-glucuronidase gene
UV	Ultraviolet
YEP	Yeast extract- peptone

Chapter 1

Introduction

1.1 General Introduction

Malting quality of barley, an economically important phenotype, is a complex, multi-component trait. Genetic improvement of malting quality is impaired by its quantitative nature and low heritability. Several studies have been undertaken to genetically dissect this trait and to localize individual quantitative trait loci (QTL) on the genetic map of barley. Chromosome 4H of barley holds two important malting quality QTLs. Amongst them, one major QTL complex – QTL2, affects several malting quality parameters including malt extract and kernel plumpness. The QTL2 was mapped on the short arm of chromosome 4H. Information regarding gene/genes associated with this QTL region is lacking. The identification of candidate genes within this QTL will lead to a better understanding of the malting quality genetics, and the development of functional markers for barley breeding.

In crop plants, map-based cloning and transposon tagging are the two most successful methods of cloning genes with distinct phenotypes. However, the 5 million kb barley genome consists of 95% non-transcribing sequences, resulting in tedious and inefficient map-based cloning. The maize *Ac/Ds* transposon system has been successfully utilized for gene cloning in heterologous plant species, like barley (Koprek et al., 2000; Singh et al., 2006; Zhao et al., 2006; Ayliffe et al., 2007). This is particularly useful for gene discovery when sequence information is not available. Recently, *Ds* transpositions in barley have been demonstrated at high frequencies over multiple generations (Singh et al., 2006) and a preference for re-insertions into closely linked genic regions (Singh et al., 2006; Ayliffe et al., 2007). These properties facilitate targeted mutagenesis utilizing *Ds* transposons. Through the reactivation of the *Ds* transposons, *Ds* loci mapping near a gene of interest are important candidates for saturation mutagenesis and gene cloning in barley (Cooper et al., 2004).

The aim of this study was to saturate the malting-quality QTLs located on chromosome 4H with *Ds* insertions, particularly at the QTL2 region. Two TNP lines – TNP-29 and -79 were identified as important candidates for saturation mutagenesis as *Ds* loci in these lines were mapped on chromosome 4H near the important malting quality QTLs. The reactivation of *Ds* transposons was performed by crossing candidate *Ds* insertion lines with an *Ac*TPase expressing line. In addition, efforts were made to device an *in-vitro Ds* activation method involving an extra-chromosomal transient expression of *Ac*TPase. In order to obtain flanking sequences in the newly generated TNP lines, a new PCR- based protocol – the high efficiency thermal asymmetric interlaced PCR (HE-TAIL PCR), was developed.

1.2 Hypotheses

1. Malting quality QTLs on chromosome 4H contains important gene or genes that affect the malting quality of barley.

2. Newly developed transposon insertion lines can be utilized for gene identification in the malting quality QTLs.

3. Maize *Ds* transposons can be reactivated *in vitro* through transient expression of transposase gene in barley embryos.

4. PCR based approaches can be utilized efficiently to isolate genomic sequences adjacent to *Ds* elements.

1.3 Objectives

1.3.1 Study 1:

Reactivation of *Ds* trasposons through conventional breeding methods

Aim 1: To reactivate *Ds* transposon linked to malting quality QTLs by crossing with a transposase expressing line.

Aim 2: To identify new *Ds* insertion lines by performing phenotypic and molecular screening.

Aim 3: To generate flanking sequences from newly identified *Ds* insertion lines.

Aim 4: To assign putative function to *Ds* flanking sequences using basic bioinformatics analysis.

1.3.2 Study 2:

Construction of new *Ds* transposon lines through *in-vitro* methods

Aim 1: To create a binary construct containing the *Ac*TPase transposase and *GFP* genes.

Aim 2: To transform candidate barley *Ds* lines with new construct for transient expression of *Ac*TPase.

Aim3: To identify new *Ds* insertion lines using molecular screening methods.

1.3.3 Study 3:

Development of new efficient strategy for generation of flanking sequences

Aim 1: To identify an efficient PCR based method for the generation of *Ds* flanking sequences.

Aim 2: To improve existing thermal asymmetric interlaced PCR (TAIL PCR) method for high throughput analysis.

Aim 3: To test the validity of newly developed HE-TAIL PCR for the isolation of genomic sequences in barley.

Chapter 2

Literature Review

2.1 Barley and its importance

Barley is amongst the first cultivated crops in the Old World, with records of cultivation from approximately 10,000 years ago. Originating from the Fertile Crescent (Zohary and Hopf, 1993), this is one of the major corps that was successfully domesticated along with other crops such as einkorn, emmer wheat, lentils, peas and chickpeas, bitter vetches and flax (Slafer et al., 2002). Archeological excavations found early remains of cultivated barley in the Nile Delta, Egypt (Darby et al., 1977). In addition, barley was credited as a gift from goddess “Isis” and germinating barley kernels symbolized the resurrection of goddess “Osiris” (Slafer et al., 2002). Therefore, barley holds immense cultural and practical significance.

Cultivated barley (*Hordeum vulgare* ssp. *vulgare)* is one of the four most important cereals worldwide and is mainly used as feed grain and malt production. It is an economically important crop in Canada and is ranked as the 3rd most produced commodity in Canada behind wheat and maize (FAO, 2007). Canada is also the 3rd largest producer of barley in the world behind Russian Federation and Spain (FAO, 2007). In 2009, 3.5 million hectares of barley was grown and a total of 9.5 million tons of barley was produced in Canada (Statistics Canada, 2010). As the 3rd largest exporter of malt, Canada exports approximately 0.7 million tons of malt in 2007 and the production of malt is projected to increase to 0.8 million tons by 2011 (Government of Alberta, 2010).

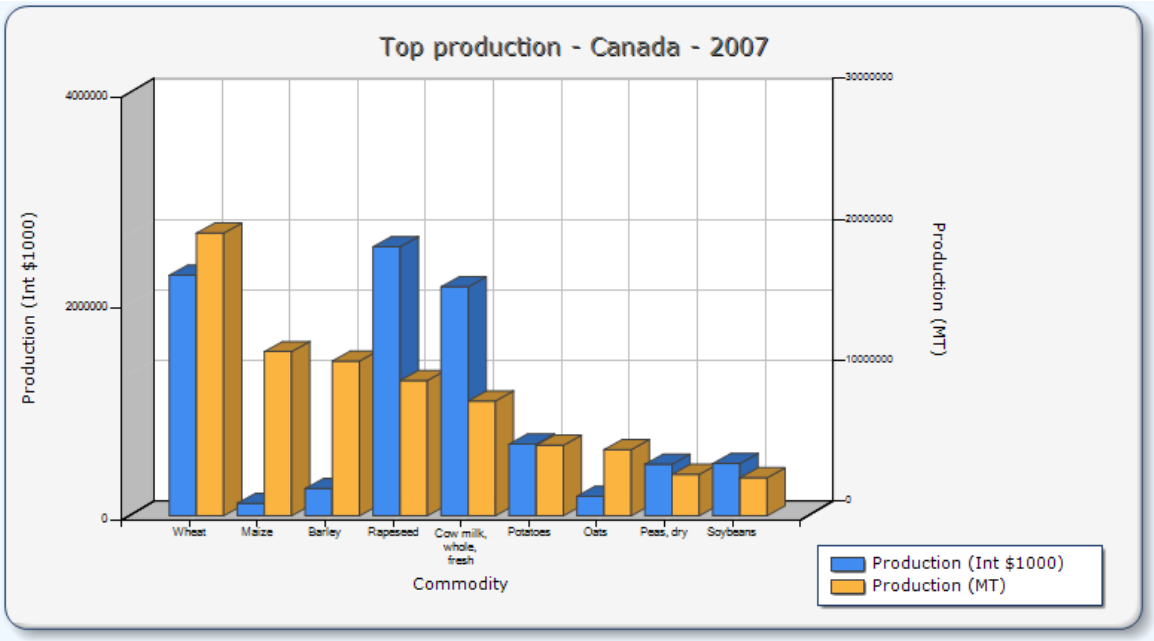

Figure 2.1. Top production of commodities from Canada. Data copied with permission: http://faostat.fao.org/site/339/default.aspx, FAOSTAT, Statistics Division, Food and Agriculture Organization of the UN.

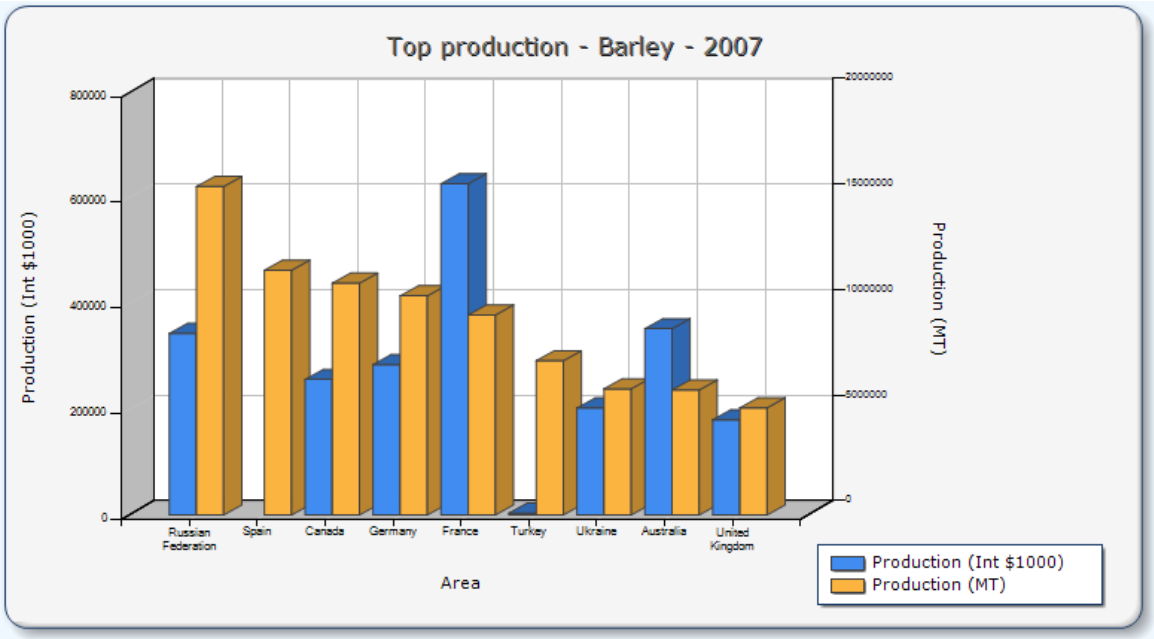

Figure 2.2. Countries ranked based on the amount of barley produced. Production of barley in Spain, in terms of value, was not available. Data copied with permision: http://faostat.fao.org/site/339/default.aspx, FAOSTAT, Statistics Division, Food and Agriculture Organization of the UN.

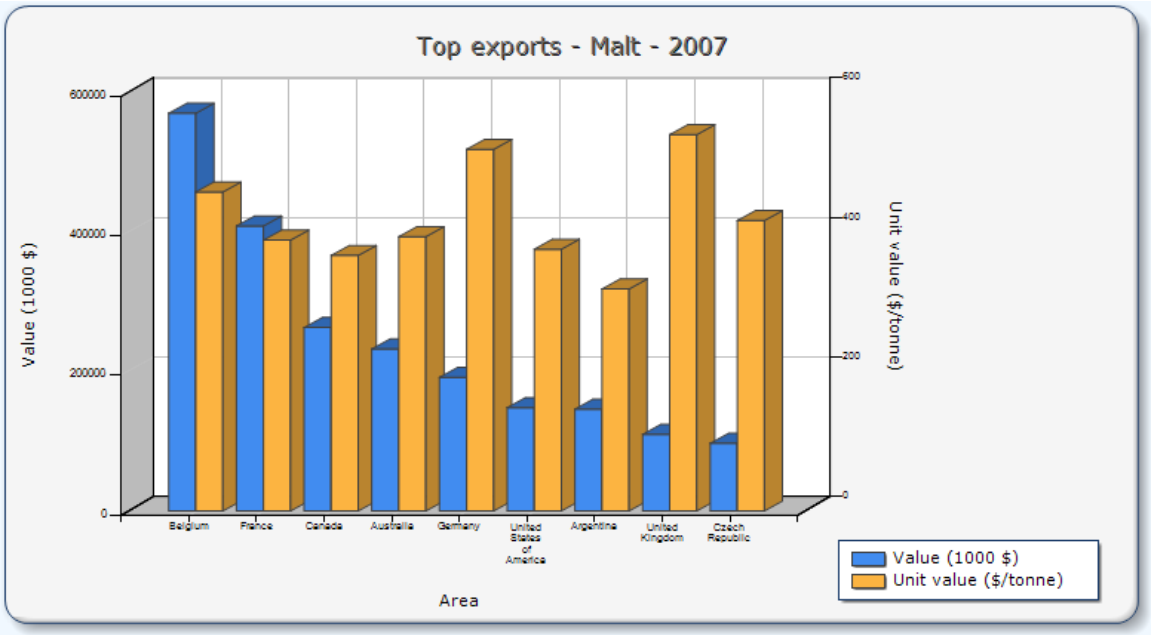

Figure 2.3. Ranking of countries based on the amount of malt exported. Data copied with permission: http://faostat.fao.org/site/342/default.aspx, FAOSTAT, Statistics Division, Food and Agriculture Organization of the UN.

As a general rule, barley is grown on marginal lands of less favorable conditions (Hillman, 1975). This was supported by archeological evidence indicating large amounts of barley grown on depleted and/or saline soils during the 4th millennium (Zohary and Hopf, 1993). Unfortunately, the use of barley as a staple food source declined since baking was introduced. Despite that, barley remains as an important crop due to its main use as animal feed and in the beer making industry. Currently, barley is the major food source in Tibet and Ethiopia due to the salinity problems faced by these countries (Baik and Ullrich, 2008).

Although wheat, rice and corn are main dietary staples in the world, over the past decade, there has been an increasing interest in barley for human consumption. This is mainly due to its content of health-related bioactive components (Manach et al., 2004). The health benefits normally associated with barley are attributed to high amounts of dietary fibers. Large-scale attempts are being made to genetically increase the level of β-glucan in barley (Baik and Ullrich, 2008). In addition, antioxidants or phenolics have also been detected in

barley (Bonoli et al., 2004; Cavallero et al., 2004). Studies have shown that barley contains more phytochemicals than previously considered (Perez-Jimenez et al., 2005; Adom and Liu, 2002). These bioactive compounds have revived the use of barley for human consumption, especially in the present health conscious environment. High abundance of antioxidants, such as the tocols family, is known to reduce serum LDL cholesterol through their antioxidant action (Qureshi et al., 1986). In addition, β- glucan found in barley was reported to lower blood cholesterol and the glycemic index (Pins and Kaur, 2006). These health properties have upgraded barley once again as an important food source in the society.

Currently, the primary use of barley is for animal feed and for malt production for the brewing and distillery industries (Bhatty, 1992). About 85% of today's world barley production is used as animal feed because barley has advantages over other feed crops such as maize. These advantages include its short vegetation period and ability to grow in harsh environments (Slafer et al., 2002).

Despite the growing use of barley as animal feed, large scale cultivation of barley is still practiced due to its major input in the brewing industry. Malt, produced from germinating barley is used for making alcoholic beverages. Brewing companies are the main consumers of barley malt. In Canada, around 2.9 million tons of beer is being produced annually (FAO, 2008). In addition, Canada is one of the major exporters of malt. It has been estimated that 0.7 million tons of malt was exported from Canada in 2007. Although beer consumption in Europe, where most brewing factories were established, is steadily declining due to increased health awareness, there is an upward trend for beer consumption in developing countries such as Brazil which ranks first as the major importer of beer made from barley (FAO, 2007).

2.2 Barley genome and resources

Barley belongs to the Poaceae family, the largest family within the monocotyledonous plants. This family includes other major cereal crops including

wheat, maize, rice, and rye, as well as important forage grasses such as ryegrass, fescue, and Kentucky bluegrass (Varshney et al., 2007). In the Poaceae family, several cereal crops belonging to the *Triticeae* tribe, such as barley, wheat, rye and triticale, are genetically closely related. However, the use of these crops varies widely, for example, pasta, beer, bread, animal feed and soup ingredients. Barley (*Hordeum. vulgare*) is a model genome system for *Triticeae* and is a self-pollinating diploid with 2n=2x=14 chromosomes. This is because barley chromosomes are homoeologous to those of cultivated wheat, oats and rye. However, other *triticeae* crops have much larger genomes than that of barley. Also, the self-fertile, diploid genetic system carries many advantages for development of homozygous material and especially for studies of gene expression. Even so, barley has large genome size of approximately 5000 Mbp (Bartoš et al, 2008; Arumugathan and Earle, 1991; Bennett and Smith, 1976) with more than 80% of the genome consisting of repetitive DNA sequences (Sreenivasulu et al., 2008). Such large and complex genomes make both genome analyses and crop improvement a challenging task (Langridge et al., 2001).

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 (Sreenivasulu et al., 2008). These resources for barley include large bacterial artificial chromosome (BAC) (Yu et al., 2000) and cDNA libraries (http://harvest.ucr.edu/BarleycDNA_Library.htm), widely used mapping populations (http://wheat.pw.usda.gov/maps.shtml#barley), extensive mapping resources (NCBI; http://www.ncbi.nlm.nih.gov/; an international genome database, GrainGenes (http://wheat.pw.usda.gov/GG2/index.shtml), large number of Expressed Sequence Tags (ESTs) representing the majority of genes (http://harvest.ucr.edu/), a BarleyChip microarray to monitor expression of 22K genes (http://harvest.ucr.edu), EMS mutant populations (Caldwell et al., 2004) and transposon insertion mutants (Singh et al., 2006). Recently, in order to provide the fundamental information regarding functional and structural aspects of barley genome, the International

Barley Genome Sequencing Consortium has been established (http://barleygenome.org/) to sequence the complete genome of barley.

2.3 Colinearity and microcolinearity

Although, barley has a large genome size, it has the smallest genome compared to other synteneous species such as wheat ~18000 Mbp (Gill et al., 2004), oats ~11315 Mbp (Arumuganathan and Earle, 1991) and rye ~7917 Mbp (Bartoš et al, 2008). Synteneous genomes contain high conservation of order within gene structures, which allows comparison of position and function of genes between species through comparative genomics. Hence, biological studies performed in other species can be applied to barley especially between synteneous regions of barley, wheat and rice.

Since rice is the first cereal crop to have its genome sequenced combined with the high synteny between the barley and wheat genomes, large amounts of data have been generated to determine the colinearity between barley, wheat and rice. Using various methods to physically map barely genes onto the rice and wheat chromosomes, researchers were able to determine regions of synteny between barley, wheat and rice. Barley chromosomes 2(2H), 3(3H), 4(4H), 7(5H), 6(6H) and 1(7H) are found to be synteneous to wheat chromosomes 2, 3, 4, 5, 6 and 7 respectively (VanDeynze et al., 1995; Gale and Devos, 1998). Also, barley chromosomes have been found to be synteneous 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 (Cho et al., 2006).

This general colinearity between barley, wheat and rice has aided in many gene discoveries, especially genes located on barley chromosome 3H (Bilgic et al., 2007) which is highly synteneous to rice chromosome 1. On a smaller scale, microcolinearity studies were done in conjunction with mapping studies to saturate small regions of the barley chromosomes (Drader et al., 2009). Examples include investigation of barley chromosomes 2H, 3H and 7H (Drader et la., 2009; Bilgic et al., 2007). Expressed sequence tags (ESTs) from synteneous chromosome regions between rice and barley were obtained from bacterial

artificial chromosomes (BACs) of rice and mapped onto the barley chromosome (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; Stein et al., 2005), and *Ppd-H1* (Turner et al., 2005) are some examples of successful utilization of microcolienarity for gene identification.

Although colineraity and microcolienarity studies can aid greatly in identifying homologues of genes, difficulties exist to discover genes from non-linear genomic regions. One such region is located on the short arm of chromosome 4H of barley. This region was found to be non-synteneous to rice due to genomic rearrangements in rice and barley chromosomes (Bilgic et al., 2007). Hence, in some instances synteny and micro-colinearity can provide only limited insight into genome organization and alternative methods have to be utilized to further understand the genome organization and function in barley.

2.4 Barley malting and its genetics

Barley is a key ingredient in the malting and brewing industry. Barley grains are used in the malting industry for the production of beer, and this was listed as the second most important use for barley grains behind animal feed (FAO, 2007). The general biochemical process in brewing is to digest barley starch into fermentable sugars that are then converted into alcohol by yeast metabolism (White et al., 2006). Malting is driven by the synchronized activity of hydrolytic enzymes and endoproteases that modify the cell walls and protein matrix, exposing the starch granules of the endosperm to depolymerization (White et al., 2006). The relative rate of protein and carbohydrate modification probably plays the most important role in the application of malt during the brewing process.

During the malting process, barley kernels are cleaned and sized, steeped, germinated and then kilned. Malting barley cultivars are distinguished from non-malting cultivars by their ability to rapidly and uniformly modify sugars by activating numerous enzymes like α-amylase and hydrolases during

germination (Ramsmusson et al., 1985). Following this, the malt is ground and extracted to form wort. Various tests of the wort are used to assess quality factors such as malt protein concentration, soluble proteins, and diastatic power, which are highly influenced by genetic and environmental factors.

Diastatic power (DP) and fermentability, also known as apparent attenuation limit (AAL) are two key parameters that are used in the brewing industry to describe barley malting quality. DP is regarded by maltsters and brewers as a measure of the combined activity of α-amylase, β-amylase, limit dextrinase, and α-glucosidase (Yang et al., 2009). In addition, numerous other parameters including malt extract yield (ME), amylase activity (AA), malt β-glucan content (BG), kernel plumpness (KP) and seed dormancy (Dor) also influence malting and brewing of barley. Efforts from barley researchers concluded that adequate levels of α- and β-amylase and β-glucanase activity, in combination with low malt β-glucan content is required for high quality malt for brewing (Swanston et al., 2002). Therefore, malting quality is a complex trait due to its dependence on many combinations of malting parameters.

The methods assessing malting quality by micromalting are time-, resource- and labor-intensive. The molecular markers offer simple, efficient and reliable approaches to identify desirable plants containing favorable alleles at loci affecting malting quality. Many types of molecular markers have been developed and are being used to track different loci and genomic regions including important Quantitative Trait Loci (QTLs) (Varshney et al., 2006; Jahoor et al., 2004; Tuberosa and Salvi, 2004).

The combination of molecular markers and conventional breeding can speed up the process of developing of an improved variety. Through molecular breeding efforts a new barley cultivar named “Tango” was developed. This variety is the first barley variety developed through marker assisted selection (MAS) to be released in the USA, and has proven to be superior to other varieties in its resistance to the stripe rust pathogen (Hayes et al., 2003). Using MAS, the time to produce a new variety have been cut short from approximately 14 years to about 7 years.

2.5 Discovering malting quality QTLs

Extensive efforts have been made to develop molecular markers for improving the malting quality of barley. Initial studies by Hayes et al. (1993) and Han et al. (1995), detected several QTLs controlling grain yield and malting quality in progenies derived from a cross of agronomically relevant germplasm. Malting quality is composed of a number of traits including percentage of malt extract, total grain protein, wort protein, β-glucan, kernel plumpness, α-amylase activity, kernel weight and diastatic power (Han et al., 1995).

At least 286 QTLs representing 17 malting quality traits from 18 separate crosses of different barley cultivars have been localized on all 7 chromosomes of barley (Wei et al., 2009b; Zale et al., 2000). Based on consensus mapping studies, 84% of malting quality QTLs have been found to be conserved among all the mapping populations. Although several QTLs that significantly affect malting quality in barley were detected on most chromosomes, a QTL with a large effect on the numerous malting quality traits was found in the *Bmyl-ksuH11* interval on chromosome 4H (Han et al., 1995). Similarly, Han et al. (1997a) identified another important region of chromosome 7H containing overlapping QTLs for malt-extract content, α-amylase activity, diastatic power, malt β-glucan content, malt β-glucanase activity and dormancy. This complex QTL region also showed the largest and the most consistent effects for these traits over multiple locations and years. The locations for these malting quality QTLs have been further confirmed in a recent study by Schmalenbach and Pillen (2009). Malting quality QTLs were identified using a set of 39 wild barley introgression lines. In addition, two QTL clusters with simultaneous effects on malting traits were mapped to chromosomes 7H and 4H which were consistent with previous studies. A large QTL representing many malting quality traits was identified in the region between MWG634 and BCD402B in the telomeric region on the short arm of chromosome 4H (Gao et al., 2004; Zale et al., 2000). This QTL complex, known as QTL2, affects several malting quality parameters, namely ME, DP, AA, BG and Dor (Hayes et al., 1993).

Malting quality QTLs are known to affect more than one phenotype as described above. This suggests the existence of gene clusters or pleiotropic gene effects, where a gene could potentially affect several parameters (Zale et al., 2000). A complex trait such as the malting quality of barley is also strongly influenced by genotype x environment interactions and gene x gene interactions. Due to the high variability caused by interaction with the environment, the cause for the difference in malt extract of barley is very difficult to determine. To understand the genetics behind these complex QTLs, molecular markers can be utilized to determine the segregation of certain malting traits with DNA polymorphisms (see review Rae et al., 2007; Ayoub et al., 2003; Collins et al., 2003a; Hayes et al., 1993). The review by Collins et al. (2003a) combined all the studies of Australian barley populations that were crossed into different backgrounds to validate regions found to be associated with malt extract. They found that there are 8 regions associated with malt extract in 3 mapping populations, and of those 8, only 4 regions are found to have an effect on malting quality when crossed into a different genetic background. A similar study was performed by Coventry et al. (2003) to assess diastatic power.

Besides providing vital information regarding DNA polymorphisms associated to a trait and genotype, molecular markers can also be utilized to incorporate important QTLs in elite breeding lines through MAS. In a recent study, researchers attempted to produce a better barley variety by selecting for good malting alleles from two malting barley populations, Harrington and Morex, using MAS. Unfortunately, the combination of superior alleles did not provide expected malting quality in the progeny (Emebiri et al., 2009). This may be due to the limitations of the current QTL maps which require further saturation with new molecular markers. Current QTL regions are large and often overlap with diverse traits. However, barley breeders require precise QTL regions in order to reduce undesired gene effects of the introduced QTL in their barley breeding line (Slafer et al., 2002). Hence, there is a need to precisely explore these QTLs regions for the gene or genes within.

Although the parameters, such as malt-extract content (ME), α-amylase activity (AA), diastatic power (DP), malt β-glucan content (BG), malt β-glucanase activity and dormancy (Dor) among many others, have been shown to correlate with malting quality, the genes present in these QTLs have not been investigated. Large numbers of new molecular markers are being developed for fine mapping of important regions affecting important malting quality traits (Rostoks et al., 2005; Gao et al., 2004). Saturated molecular maps and fine mapping of QTLs will be the first step towards the identification of genes harbored in these QTLs. Once the gene or genes in the malting QTL has been determined, the sequence of the alleles responsible for the phenotype can be used as a functional marker for the development of better barley varieties.

2.6 Fine mapping of malting QTLs in barley

Malting quality QTLs in barley were not explored extensively for identification of gene/genes. Instead, fine mapping of QTLs was pursued to identify reliable molecular markers. As discussed above, many related malting quality genes occur together due to either pleiotropic gene effects or the presence of gene clusters (Zale et al., 2000). The same was true for the QTLs discovered by Han et al. (1995) using a Steptoe/Morex double haploid population. Although these authors were interested in QTLs that affect malt β- glucan and β-glucanase, they observed that the same regions also affected many other parameters of malt quality. Hence, they named these QTL regions for malting quality on chromosomes 1(7H) and 4(4H) as QTL 1 and QTL 2 respectively. Based on consensus markers, QTL 2 was identified between markers MWG634 and BCD402B in the telomeric region on the short arm of chromosome 4H.

Since then, several studies have been conducted to study these QTL regions (Gao et al., 2004; Han et al., 2004; Han et al., 1997b). The QTL2 region, originally mapped to the short arm of chromosome 4(4H), spanned about 30cM (Han et al., 1993). However, Gao et al. (2004) have successfully refined its mapping position to only 15.8cM between the MWG634 and CDO669 markers (Figure 2.4). The huge difference in mapping the QTL region may be due to the

availability of markers and methods employed. Han et al. (1997b) used molecular markers to identify QTLs in populations that were derived from only one recombination event, causing the QTL mapped to be a large region on the chromosome. However, Gao et al. (2004) used a reciprocal-substitution mapping approach by crossing Steptoe and Morex and backcrossing into the parental background to fine-map the QTL.

Although mapping procedures saturated the QTL region with additional molecular markers, information regarding gene/genes present in the malting quality QTLs present on chromosome 4H is still lacking. Hence, the aim of the proposed study was to focus on two QTLs located on chromosome 4H, QTL2 located on the short arm described by Gao et al. (2004) and another unnamed QTL located on the long arm described by Schmalenbach and Pillen (2009) for identification of important genes that are contained within this region.

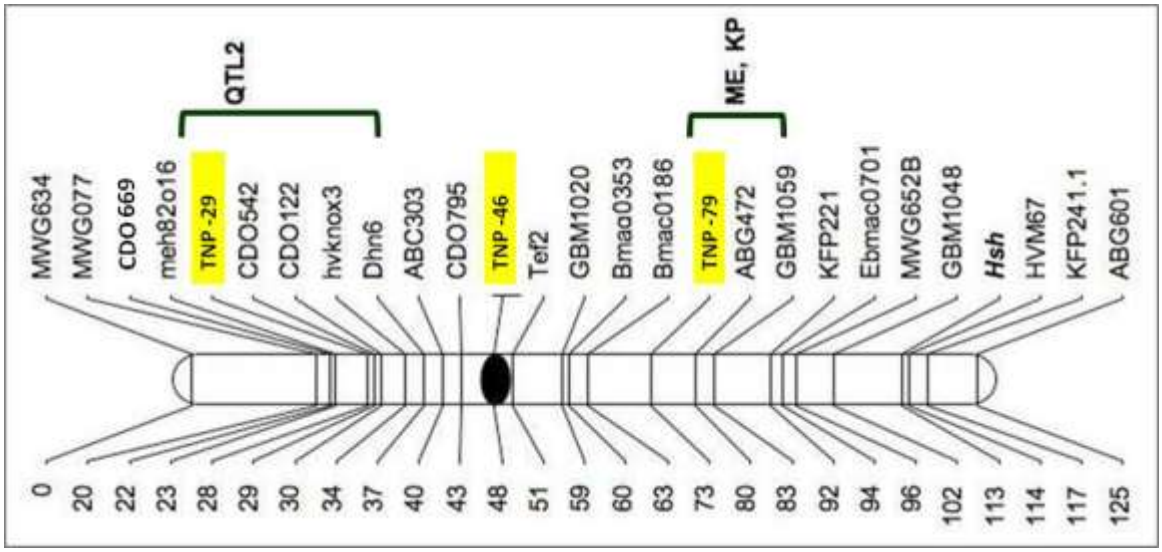

Figure 2.4. Chromosome 4H of barley showing important malting quality QTLs. QTL2 is located on the short arm of chromosome 4H at 0cM to 30cM. Another unnamed malting quality QTL is located on the long arm of chromosome 4H between 80cM and 95cM. The loci highlighted in yellow indicate the location of *Ds* transposons relative to some important malting QTLs, these transposons will be explained in the subsequent sections.

2.7 Malting quality genes

Many techniques have been employed to understand the genes affecting malting quality. Using cDNA array, studies have been conducted to determine genes that are involved in malting quality (Lapitan et al., 2008; Potokina et al., 2004). As many as 30 candidate genes have been identified for each of the six malting parameters based on ten barley genotypes are believed to have superior malting quality (Potokina et al., 2004). Some of the genes identified through these studies have been found to be located in the regions where malting quality QTLs have already been identified. Recently, Lapitan et al. (2008) studied differentially expressed genes during malting using Barley1 GeneChip array. The correlation of expression profiles with malting quality phenotypes resulted in the identification of more than 200 genes showing correlation with four malting quality traits. Although these studies do not provide definitive information regarding the function of these genes, they will be able to direct barley researchers for their experiments to uncover the mystery genes in these malting quality QTLs.

In addition to using a cDNA array, White et al. (2006) have recently created LongSAGE tag libraries from malting barley grain samples. This study identified many genes that play important roles in the germination of barley kernels. These identified tags include transcripts responsible for stress response and cell defense, protein synthesis, cell structure, plant growth and metabolism (White et al., 2006). Eight transcripts were identified as important enzymes in barley germination: α-amylase type B, α-glucosidase, (1-3,1-4)-β-D-glucanase, isocitrate lyase, β-1,3-glucanase, pyruvate kinase-like protein, xyloglucan endotransglycosylase-like protein and cysteine proteinase EP-B.

Combining all the information from the studies described above, Druka et al. (2008) developed GeneNetwork (http://www.genenetwork.org/), an online public software for the analysis of systems biology. This software was developed by integrating barley genotypic, phenotypic and mRNA abundance data sets for various traits including malting quality traits. This electronic network has the potential to identify and substantiate gene targets for saturation mapping and positional cloning.

Apart from the mass of information regarding malting quality genes generated by various laboratories, more notable barley malting genes such as α-amylase (*Amy*), β-amylase (*Bmy*) and β-glucan (*GSL*) have been extensively studied (Lukhanina et al., 2009; Matthies et al., 2009). These gene products have been found to be involved in the production of good quality malt. α-amylase and β-amylase are two important enzymes that catalyses the last step of producing maltose for the subsequent fermentation to produce malt (Wei et al., 2009a) therefore, acting as a measure for the quality of malt. Recently, the roles of other starch degrading enzymes, α-glucosidase and limit dextrinase in relation to malt quality has been explored (Lapitan et al., 2009). Together, these starch degrading enzymes form a parameter known as the diastatic power (DP), an important determinant of malt quality (Yang et al., 2009).

While an increased expression of *Amy* and *Bmy* genes are favorable for the production of high quality malt, increased quantity of β-glucan is not preferred. β-glucan, also known commonly as callose, is encoded by the enzyme callose synthase (GSL) (Schober et al., 2009) and is the major component in the pectin layer of the cell wall (Fincher, 2009). Therefore, the presence of high amounts of β-glucan will decrease the ability of cell wall/starch degradation. Due to the major role of β-glucan in cell wall production, it has prompted an increased interest in the malting industry and also for the Bio-fuels community. Efforts have been made to identify the callose synthase genes, but these efforts have been largely unsuccessful (Fincher, 2009).

2.8 Functional genomics efforts in barley

Many functional genomics tools have been developed for gene identification in barley and other cereals. These methods include generation of mutants, ESTs, physical mapping, sequencing and bioinformatic analysis. Large scale sequencing programs for the development of expressed sequence tags (ESTs) from various cDNA libraries have been initiated (Sreenivasulu et al., 2008). To date, a total of 501,366 ESTs covering different cDNA libraries from various stages of plant development and tissues are available (NCBI-dbEST

summary). Comprehensive analysis of extensive EST resources generated from barley genome facilitates gene discovery programs (Sreenivasulu et al., 2008; Zhang et al., 2004).

In addition, physical maps represent an important link to connect sequences to genetic information (Sreenivasulu et al., 2008). High resolution physical maps of all seven barley chromosomes have been prepared. Using microdissection techniques, translocation breakpoints of individual chromosomes were isolated. These translocation breakpoints are the chromosomal segments that break off and rejoin onto non-homologous chromosomes during reciprocal translocation (Mahama et al., 2002). Based on the relative translocation breakpoints, DNA markers from both genomic as well as gene-based sequences were derived (Kunzel et al., 2000).

In order to determine gene-function relationships, large scale genome-wide forward and reverse genetics approaches have been developed in barley (for review, see Waugh et al., 2006). Most of these approaches involve generation of mutants and screening for phenotypes. For example, techniques such as targeted induced local lesions in genome (TILLING), transposon mutagenesis, protein engineering etc. were developed to explore the genome of barley (Singh et al., 2006; Waugh et al., 2006; Cladwell et al., 2004; Koprek et al., 2002). Chemical mutagenesis, such as TILLING, was shown to be very efficient in creating large numbers of plants with genetic diversity in a short amount of time (Cladwell et al., 2004). Unfortunately, this method is not suitable for creating mutations to saturate a particular region such as a QTL. This is due to the general properties of chemical mutagenesis. Mutations occur randomly and cannot be directed to a specific site on a genome. In addition, all genes are not equally accessible to EMS mutagenesis. More importantly, proteins with point mutations generated through TILLING are generally functional, which will not be useful for gene function analysis.

Another method to identify a QTL would is to micro dissect a chromosome region. This method can be coupled with expressed sequence tag (EST) analysis and was demonstrated by Jiang et al. (2009). Although this is a

very crude method whereby a section of the chromosome is estimated and excised for analysis, this method has several benefits compared to ESTs because expressed sequences obtained by this technique are larger in size in addition to having 3' end information. Successful utilization of this method will also provide an improved method for genomics and functional genomics research in polyploidy species or species with large genomes, especially for EST development and mapping. Although ESTs provide some information regarding the putative roles of genes, it cannot be solely used to clone genes.

Among the many methods mentioned above, functional genomics in crop plants are mainly performed by map-based cloning and transposon tagging. Both these methods are most successful for cloning genes with distinct phenotypes. The haploid barley genome is approximately 5 million kb where most of the sequence is non-transcribing, therefore, causing map-based gene cloning tedious and inefficient. Despite this, several key genes were isolated using map-based cloning in barley.

2.9 Map-based cloning in barley

Map-based cloning, also known as positional cloning, requires the development of high-density genetic maps and the possibility to perform chromosome walking on large genomic fragments (Feuillet et al., 2003). The first success of map based cloning dates back to 1997 when a disease resistance gene (*mlo)* in barley was cloned (Buschges et al., 1997). Construction of BAC library from the barley cultivar Morex (Yu et al., 2000) greatly facilitated the positional cloning of two other powdery mildew genes named *Mla1* and *Mla6* (Halterman et al., 2001; Zhou et al., 2001) and the stem rust resistance gene *Rpg1* (Brueggeman et al., 2002). Recently, a domestication gene (*vrs1*) involved in the development of six row spikes in barley has been isolated through map-based cloning (Komatsuda et al., 2007). Despite the recent success of isolating genes using map-based cloning, this method is generally limited to small genomes. Positional cloning has remained very tedious and inefficient in large (>5,000 Mb) and repetitive (>80%) genomes such as those of barley and wheat

(Peters et al., 2003). An alternative gene cloning approach is based on transposon mutagenesis. This method offers great potential for efficient functional analysis and cloning of genes especially in large genome cereals (Singh et al., 2006).

2.10 Transposon mutagenesis

Since the discovery of the “jumping genes” by McClintock (1941), researchers have confirmed the existence of these genes in many other species, ranging from the plant to the animal world. There are many different types of transposons which are mainly classified in two major categories. The first category is the class I transposons, also known as retrotransposons. These transposons transpose through an RNA intermediate generated by reverse transcription. The second category of transposons is the class II transposons. These transposons are DNA based transposons that transpose through DNA cut-and-paste type mechanisms (Appels et al., 2003).

In maize, the *Ac/Ds* or the *Activator* and *Dissociation* elements were the first transposons observed by Barbara McClintock (Jones, 2005). Other maize tansposons include the *Mutator* or *Mu* element and the *Spm/dSpm* or the *Suppressor-mutator* transposons. Among the various types of class II transposons, the *Ac/Ds* system has been widely used for mutagenesis and saturation mutagenesis in plants. This is due to the well understood properties and mechanisms of transposition. *Ac* is a complex genetic element that is 4563 bp. It consists of an 11 bp terminal inverted repeats (TIRs) at the 5’ and 3’ end (Jones, 2005) and a transposase (*Ac*TPase) gene that encodes for an enzyme of 807 amino acids (Kunze and Starlinger, 1989). This enzyme recognizes the 11 bp TIRs of the transposon and functions to splice the genome at the site of these 11 bp inverted repeats. Besides the 11 bp TIRs, another important region essential for the transposition is the sub-terminal region of 250 to 300 nucleotides on both ends (Varagona and Wessler, 1990; Coupland et al., 1988).

While *Ac* contains the crucial *Ac*TPase transposase enzyme, the *Ds* encodes for a mutated or non-functional transposase. When *Ac*TPase is

expressed, the *Ds* is able to jump due to the presence of the TIRs and the sub-terminal regions of 200 to 250 nucleotides on both ends of the *Ds* transposon (Varagona and Wessler, 1990; Coupland et al., 1988). Therefore *Ds* is known as the non-autonomous element due to its inability to transpose in the absence of *Ac*TPase. *Ac* also contains the TIRs and sub-terminal regions allowing it to transpose to other regions in the genome by itself. Therefore, *Ac* is known as an autonomous element. Other properties of the *Ac/Ds* transposons include the generation of 8bp duplications during the insertion of the transposon and the presence of "foot prints" during the excision of the transposon. During insertion of either *Ac* or *Ds*, these 8 nucleotides are duplicated (Pereira, 1998). When the transposon excises, the complete or partial 8 base pair duplication is left behind, thus leaving a "foot print" at the site of excision. Footprints are very useful to analyze the site of insertion by the transposon (Singh et al., 2006).

2.11 Transposon tagging and gene isolation

The use of transposons for plant functional genomics in heterologous species began when the maize transposable element *Ac* was transformed into tobacco (Baker et al., 1986). The successful demonstration of *Ac* to transpose in the tobacco genome has paved the way for the utilization of transposons in heterologous systems (Yoder et al., 1988; Baker et al., 1986; Fedoroff, 1983). This demonstration led to the development of one- and two-element transposon tagging resources in many plant species including dicots, such as *Arabidopsis* (Bancroft and Dean, 1993; Van Sluys et al., 1987) and tomato (Cooley et al., 1996; Jones et al., 1994), and monocots, such as rice (*Oryza sativa* L.) (Kolesnik et al., 2004; Upadhyaya et al., 2002; Nakagawa et al., 2000; Shimamoto et al., 1993) and barley (*Hordeum vulgare* L.) (Singh et al., 2006; Scholz et al., 2001; Koprek et al., 2000).

The use of transposable elements in barley began when McElroy et al. (1997) used a simple transient assay in which *Ac*TPase was introduced simultaneously with a uidA (*b*-glucuronidase) gene disrupted by *Ds*. They successfully demonstrated the excision of the maize transposon *Ds* in barley

scutellar tissues. This success was followed by a subsequent study to introduce the maize *Ac/Ds* elements into Golden Promise, a barley cultivar, through particle gun bombardment (Koprek et al., 2000). *Ac*TPase and *Ds* elements from maize were shown to be stably transformed into barley. Scholz et al. (2001) has also reported a successful introduction of maize autonomous element, *Ac* in barley. A two-element, *Ac/Ds*-based transposon tagging system was tested by crossing transgenic *Ds* and *Ac*TPase plants. New *Ds* insertions in the F_2 generation were identified, proving the successful utilization of this system (Ayliffe et al., 2007; Singh et al., 2006; Koprek et al., 2000). The behavior of *Ds* element was systematically studied by remobilizing the elements over multiple generations to develop primary, secondary, tertiary and quaternary transpositions (Singh et al., 2006). To date, ~400 *Ds* insertion lines (TNPs) have been generated in barley (Randhawa et al., 2009; Singh et al., 2006; Zhao et al., 2006). Using a sequencing-based approach, 19 TNPs were placed at various loci on six of the seven barley chromosomes (Cooper et al., 2004). An additional 100 independent *Ds* insertion sites were also placed on the barley genetic linkage map using a PCR-RFLP approach (Zhao et al., 2006). As of 2009, flanking sequences for 80 unique *Ds* insertion lines (Singh, unpublished) have been determined. In addition, 45 of those *Ds* insertion lines have been successfully mapped onto genetic and physical maps of barley and wheat respectively (Singh, unpublished; Randhawa et al., 2009; Cooper et al., 2004). The flanking sequences and mapping data of these TNP lines is publicly available at http://wheat.pw.usda.gov/BarleyTNP/IMap/ and seeds for these *Ds* transposon (*DsT*) insertion lines are available upon request from the website.

Transposon-mediated insertional mutagenesis has several advantages over other functional genomics approaches for determining gene function. Most DNA-type transposable elements, including *Ac/Ds*, preferentially insert into genic regions. The distribution of *Ac/Ds* transpositions in maize (75%), rice (72%), Arabidopsis (64%) and barley (86%) occur in or around genes (Singh et al., 2006; Kolesnik et al., 2004; Kuromori et al., 2004; Cowperthwaite et al., 2002). This property is important especially for large genome species, like wheat, barley and

maize, where genes comprise only a small percentage (1-15%) of the genome (Wicker et al., 2005; Sandhu et al., 2002). In addition, the *Ac/Ds* transposons have a tendency to transpose to closely linked sites (Ayliffe et al., 2007; Upadhyaya et al., 2002; Parinov et al., 1999; Dooner and Belachew, 1989). This provides significant advantages for targeted mutagenesis of closely linked genes. Therefore, reactivation of *Ds* can potentially generate a wide diversity of tagged alleles. These alleles can be subsequently used to generate an allelic series or deletions of clustered genes.

Another important feature of the two-component *Ac/Ds* system is that *Ds* can be mobilized or immobilized as required, leading to better control of the mutation process. For difficult-to-transform crops, this approach requires only a few initial transformants to generate large numbers of plants with *Ds* trasposons (*DsT*) insertions at different locations. The selective reinsertion of *Ds* transposable elements in geneic regions is most important in deciphering the genome of large genome species. This is especially true for the *Triticeae* family in which many species are economically important crops with large genomes. Further analysis of these barley lines have confirmed the ability of the *DsT* to reactivate over multiple generations by crossing with *Ac*TPase lines (Singh et al., 2006). However, in rice, *Ac/Ds* transposon insertions are found to move less frequently over generations, this may be due to epigenetic regulation, causing the methylation of these transgenes (Chin et al., 1999).

Functional genemoics can be done through the *Ac/Ds* transposon tagging system. Several important genes were identified by this method (for review, see May and Martienssen, 2003). These include *Curly leaf* (Goodrich et al., 1997), *Fruitfull* (Gu et al., 1998), *Medea* (Grossniklaus et al., 1998) and *Sporocyteless/Nozzle* (Yang et al., 1999) in *Arabidopsis*, the *Knotted1* (Hake et al., 1989) and *Opaque2* (Schmidt et al., 1997) in Maize, *Branched floretless* and *Anther Indehescence1* (Zhu et al., 2003, 2004) in rice, and more recently, the *uroporphyrinogen III synthase* (*Uros*) (Aylife et al., 2009) in barley. These genes were isolated through random tagging approach where large populations of transposon-containing plants screened for mutant phenotypes. The tendency of

Ac/Ds to transpose locally (Upadhyaya et al., 2002; Parinov et al., 1999; Smith et al., 1996) can be utilized to tag a gene of interest when a *Ds* element is placed near it. Examples describing the localized tagging approach using *Ac/Ds* transposons include the targeted mutagenesis of the *pink scutellum1/viviparous7* (*ps1/vp7*) locus (Singh et al., 2003), the *sesquiterpene cyclase* gene (*stc1*) (Shen et al., 2000), *indeterminate1* (Colasanti et al., 1998) *and tasselseed2* (DeLong et al.,1993) in maize.

Using the stable transformation of a two component transposable element system, stable barley *Ds* insertion lines have been created as valuable resources for saturation mutagenesis of genetically linked genes. Amongst the single copy *Ds* insertion barley lines (TNP) developed by Singh et al. (2006), there are three lines (TNP-29, TNP-46, TNP-79) that are located on chromosome 4(4H) and two of them are closely linked to malting quality QTLs. In the case of TNP-29, the *Ds* is located in close proximity to the proposed QTL2 region by Gao et al. (2004) and is within the original QTL2 region proposed by Han et al. (1997b). As discussed before, QTL2 is one of the important malting quality QTL. Hence, this QTL was the main focus of this research. The other TNP line that was also utilized is TNP-79. The TNP-79 *Ds* insertion is located in vicinity the proposed malting quality QTL region of 80cM to 95cM by Schmalenbach and Pillen (2009). This malting QTL is located on the long arm of chromosome 4H and influences malting quality parameters including malt extract and kernel plumpness.

These TNP lines are excellent candidates for reactivation and to “walk” into linked regions of the chromosomes, preferably into the malting QTL region. By identifying the flanking sequences of these insertions, the location of newly transposed *Ds* element can be mapped onto the genome. Thus, due to the *Ds* transpositions, the QTL2 region can be saturated and candidate genes involved in malting quality can be determined.

2.12 Activity of transposase (*Ac*TPase)

Although there are many benefits of using *Ac/Ds* transposon system, there are some difficulties to be resolved. It has been observed that the expression of the *Ac*TPase in transgenic plants was dramatically reduced or turned off in advanced generations (Singh unpublished; Koprek et al., 2000). Earlier studies in rice also confirmed that loss of or reduced *Ds* activity during generation advance (Upadhyaya et al., 2002; Nakagawa et al., 2000). This was supported by the observation of the single-component *Ac* in rice that underwent a pronounced reduction in mobility due to epigenetic silencing. Therefore, the reduced *Ds* activity can be explained by the reduced expression of *Ac*TPase gene. In a different study, it was observed that the level of genomic cytosine methylation increased in dedifferentiated cell cultures containing *Ac* insertions (Kohli et al., 2004).

Also, the reduced activity of *Ac*TPase may be due to the presence of multiple copies of the *Ac*TPase gene in the transgenic plants. Since transgenic plants containing *Ac*TPase have been generated using biolistic methods (Lemaux et al., 1996), it has been shown that there are many *Ac*TPase copies present in the genome of the transformed barley plant. Studies have showed that transgene silencing occurs in transgenic plants that contain high transgene copy number (Meyer, 1998).

Complications of transgene silencing during generation advance or by high copy numbers of transgene have to be addressed. Methods such as genetic transformation using biolistcs or *Agrobacterium* methods can be used to generate new *Ac*TPase expressing plants. However, the efficiency of these methods is still relatively low in monocots, despite a recent report of high frequency transformation which has yet to be reproduced (Hensel, 2009).

2.13 Extra-chromosomal activation of *Ds*

Extra-chromosomal activity of the *Ac*TPase could be very useful to generate new stable *Ds* transpositions that are devoid of the *Ac*TPase gene in the first generation. This will make the *Ac/Ds* system highly efficient. Although extra-chromosomal gene activity has not been extensively studied in plants, this

system was shown to be successful in *Drosophila* (Karess and Rubin, 1984). A non-autonomous P element containing a gene encoding for *rosy*, a gene responsible for red pigmentation in the eye color, and another construct containing the transposase but without the terminal inverted repeats (TIRs), termed “wings clipped” plasmid, was constructed. These two constructs were co-microinjected into the *Drosophila* embryos which have a defective *rosy* gene, ie. brown eye phenotype, and was screened for red eyes. Karess and Rubin (1984) observed the successful transformation of the *Drosophila* embryos through extra-chromosomal expression of the “wings clipped” P element. The transformation frequency was about 14%. Since then, this method of transgenesis is routinely used for many experiments in *Drosophila* (Boylan et al., 2008; Choi et al., 2008).

In the plant science realm, there has only been one report on transient expression of a transgene. This experiment was done in rice by Upadhyaya et al. (2006). They have infected transgenic rice containing a *Ds* insertion with *Agrobacterium* containing the *Ac*TPase and the green fluorescent protein gene (*GFP*) as a visual marker. Their success in utilizing transient expression of *Ac*TPase for the reactivation of the *Ds* transpososn was demonstrated by obtaining new *Ds* insertion lines at the frequency of 9-13%. This report has established the possibility of performing the extra-chromosomal expression of *Ac*TPase in barley to activate the *Ds* transposon.

2.14 Detection of transiently expressing *Ac*TPase

Transient expression of a gene refers to an increased expression of a particular gene followed by a declined expression over time (Dhillon et al., 2009). The transient expression of a gene is thought to be dependent on several factors. These factors include the timing of transgene integration (Prols and Meyer, 1992), transgene copy number (Svitashev et al., 2002; Pawlowski and Somers, 1998), cell death and loss of DNA (Weld et al., 2001; DeBuck et al., 1998; Hunold et al., 1994) and also the RNA silencing or post-transcriptional gene silencing (PTGS) (Chiera et al., 2008; Butaye et al., 2005).

A transient expression of an extra-chromosomal gene has been shown to be successful in a preliminary study in gene silencing (English et al., 1997). These authors showed that the extra-chromosomal DNA encoding for GUS has been transcribed as mRNA in tobacco and successfully silenced a *GUS* gene that was present in the tobacco genome. Although, this study did not show translation of the mRNA, it has indicated the potential of a transient expression of an extra-chromosomal DNA in a cell.

In barley, extra-chromosomal activity of *Ac*TPase has not been extensively studied. However, McElroy et al. (1997) have successfully demonstrated the excision of the maize *Ds* transposon in barley scutellar tissues, using a simple transient assay. In his experiment, a uidA (β-glucuronidase) gene disrupted by *Ds* was co-transformed with *Ac*TPase and excision of *Ds* was confirmed by obtaining many GUS foci. Using similar approach, Takumi et al. (1999) observed GUS expression after *Ds* excision when wheat *Ac*TPase lines were bombarded with plasmids having a *Ds* element located between rice *Act1* promoter and a *GUS* gene.

In a more recent study by Dhillon et al. (2009), transient expression of GFP was tracked in lima beans. Their aim was to determine gene silencing suppressors in order to improve methods to determine the performance of a gene or promoter (Yoo et al., 2007). Constructs containing gene silencing suppressors were either co-introduced into lima beans with a separate GFP construct or fused to the GFP before introduction using biolistic methods. It was observed that the transient expression of GFP is at its maximum at 1.5 to 3 days post inoculation (dpi) followed by a rapid decline within 6 dpi.

Connecting Statement

Based on the comprehensive review, two major malting quality QTLs of barley are located on the chromosome 4H. With current available resources, we aim to perform saturation mutagenesis on these QTLs using the *Ac/Ds* transposon system.

In this experiment, TNP-29 and -79 were selected from a repository of stable *Ds* insertion lines (TNPs), because they are located in the vicinity of the malting quality QTLs on chromosome 4H. The reactivation of these lines was performed through conventional breeding methods by crossing TNP-29 and -79 with *Ac*TPase expressing lines. Since the *Ds* transposon tends to re-insert near the original site of insertion, the generation of new *Ds* insertion lines can be utilized for saturation mutagenesis of the malting quality QTLs on chromosome 4H.

I have contributed to all of the work pertaining to the following chapter. This includes the herbicide and molecular screening for the F_2 population, the generation of flanking sequences from new *Ds* insertion lines and the basic bioinformatics analysis of flanking sequences. Based on PCR analyses, this study has identified 46 new TNP lines which will be further confirmed for redundancy and copy number. As the QTL2 region is quite large, development of large numbers *Ds* insrtions or TNP lines will be continued through other research studies. Expansion of this study will lead to develop a better understanding of malting quality traits associated with QTL2.

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Chapter 3

Transposon mutagenesis to target malting quality QTLs

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3.1 Abstract

Malting quality, a complex multi-component trait, determines the quality of beer made in the brewing industry. Molecular mapping studies in barley indicated two major malting quality QTLs on chromosome 4H that affects several malting quality parameters. For the better understanding of these important QTL regions, the maize *Ac/Ds* transposon based mutagenesis system was exploited. The combination of the *Ds* transposon properties, to reactivate over multiple generations and the preference of re-inserting near the original site of excision and into genic regions, was utilized for saturation mutagenesis of these malting quality QTLs. From the barley mutant repository of *Ds* insertion lines (TNPs), TNP-29 and -79, mapping near the malting quality QTLs, were selected for sequential reactivation by crossing with *Ac*TPase expressing line. New TNPs were generated to identify, tag, and determine genes and their functions. Using phenotypic and molecular screening methods of herbicide screening, PCR analysis and Southern blotting, the *Ds* reactivation frequency was determined to be approximately 10% from a total of 452 plants. Basic bioinformatics analysis of 10 new flanking sequences generated through iPCR has indentified 6 sequences with unique genes. These newly developed *Ds* insertion lines can facilitate the effort of saturation mutagenesis with *Ds* transposons for a better understanding of malting quality traits and candidate genes displaying quantitative variation.

3.2 Introduction

Barley is an economically important crop and is the main cereal crop for producing malt, a product for the brewing industry. Malting quality is a complex trait, controlled by many parameters including malt extract percentage (ME), α-amylase activity (AA), diastatic power (DP), β-glucan content (BG), kernel plumpness (KP) and dormancy (Dor) (Swanston et al., 2002). These parameters are often found associated with regions of chromosomes known as quantitative trait loci (QTL). On the Chromosome 4H of barley, two malting quality QTLs were identified by Hayes et al. (1993) and Schmalenbach and Pillen (2009). The malting quality QTL2 on chromosome 4H identified by Hayes et al. (1993) in

combination with another malting quality QTL on chromosome 7H, accounts for approximately 30% of variation in malting quality. Since then, efforts have been made to saturate malting quality QTL regions with molecular markers. In this study, efforts have been made to saturate two malting quality QTLs on chromosome 4H with *Ds* transposons.

Transposons have been widely used for functional genomics to clone genes of interest through transposon tagging. Some notable examples of genes cloned through transposon tagging include *curly leaf* (Goodrich et al., 1997) in Arabidopsis, *knotted1* (Hake et al., 1989) in maize, and *branched floretless* and *anther indehescence1* in rice (Zhu et al., 2003, 2004). These genes were isolated through a random tagging approach where large populations of transposon-containing plants were screened for mutant phenotypes. The tendency of the *Ac/Ds* transposons to transpose locally (Upadhyaya et al., 2002; Parinov et al., 1999; Smith et al., 1996) can be utilized to tag a gene of interest when a *Ds* element is placed near it. Examples describing the localized tagging approach using the *Ac/Ds* transposons include the targeted mutagenesis of the *pink scutellum1/ viviparous7* (*ps1/vp7*) locus (Singh et al., 2003), the *sesquiterpene cyclase* gene (*stc1*) (Shen et al., 2000), *indeterminate1* (Colasanti et al., 1998) and *tasselseed2* (DeLong et al., 1993) in maize.

A two-component *Ac/Ds* transposable element system was introduced into barley (Koprek et al., 2000; Singh et al., 2006) for targetted saturation mutagenesis. Several single copy *Ds* insertions (TNPs) in different barley lines have been developed. Among a collection of these lines, *Ds* transposon (*DsT)* loci from TNP-29 and -79 were located on chromosome 4H (Cooper et al., 2004). The *DsT* locus from TNP-29 has been found to be in the close proximity of malting quality QTL, QTL2. Similarly, *DsT* locus from TNP-79 was assigned to long arm of chromosome 4H near another malting quality QTL. Because the *Ds* transposon tends to re-insert into genic regions that are closely linked to the original site of excision (Koprek et al., 2000; Ayliffe et al., 2007), we reactivated the *Ds* insertion lines, TNP-29 and -79, to saturate the malting quality QTLs with *Ds* transposons on chromosome 4H.

In this study, several new *Ds* transposon lines were generated through conventional breeding methods by crossing TNP-29 and -79 with *Ac*TPase expressing line (25-B). From the F_2 population of these crosses, phenotypic and molecular screening was conducted to identify new *Ds* insertion lines. Flanking sequences for these lines were generated through inverse PCR (iPCR) followed by basic bioinformatics analysis to determine the putative gene functions of these lines.

3.3. Materials and methods

The general overview of experimental method is shown in Figure 3.1.

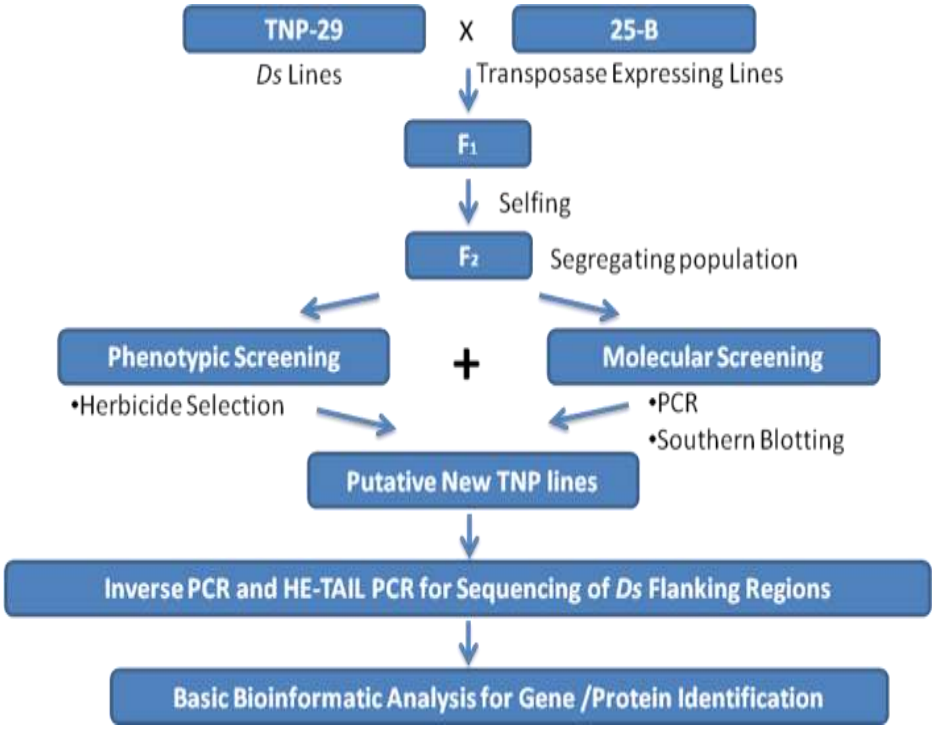

Figure 3.1. Strategy for the development of new TNPs.

3.3.1 Plant growth conditions

The maize *Ds* and *Ac*TPase transposons were introduced into Golden Promise (GP), a barley malting variety, through genetic transformation (Koprek et al., 2000). A total of 100 single copy *Ds* insertion lines were identified previously (Singh et al., 2006). The seeds of TNP-29, TNP-79 and 25-B were obtained from

the TNP repository at USDA, Aberdeen, Idaho (Singh et al., 2006). TNP-29 and -79 both contain a single copy *Ds* insertion, whereas 25-B is the transposase expressing line (Singh et al., 2006). These seeds were planted in the greenhouse with the following conditions: approximate day temperature of 65°F or 18°C and night temperature of 60°F or 15°C, and approximately 16 hours of daylight at 1000 µE. A mixture of ½ promix and ½ soil were used to plant the seeds in 6-inch pots. Two seeds were sown per pot and watered once every other day. Standard fertilizer, 20N-20P-20K, was applied biweekly to the plants.

3.3.2 Reactivation of *Ds* element in TNP-29 and TNP-79

Florets of TNP-29 and TNP-79 were emasculated by removing the anthers with a fine forceps and covered with glassine bags to prevent cross-pollination. 3-6 days after emasculation, pollen from transposase expressing line 25-B were dusted onto the individual stigmas in the florets and covered with glassine bags. The reciprocal crosses were also made by pollinating 25-B plants with TNP-29 and TNP-79 pollen. Seeds produced from these crosses were collected, grown and allowed to self pollinate to obtain F_2 seeds for further analysis described below. Seeds of F_2 population were also obtained from the TNP repository at USDA, Aberdeen, Idaho.

3.3.3 DNA extraction

A standard phenol chloroform DNA extraction protocol was used (Singh et al., 2006). Young leaves (3-4 leaf stage) from the F_1 and F_2 generation of the different crosses were collected using the snap freezing method. Leaves were then individually placed in a labeled 1.5 ml microcentrifuge tube and immediately immersed into liquid nitrogen after harvesting. Leaf samples were then stored in a -80°C freezer until DNA extraction.

For DNA extraction, leaf samples were ground in liquid nitrogen and suspended in the natural extraction buffer and SDS. This slurry was then incubated at 65°C before the addition of potassium acetate. The supernatant was treated with 1:1 phenol:chloroform to further purify the DNA. Isopropanol was

added into the supernatant and placed into the -20°C freezer to precipitate the DNA. The DNA precipitate was washed with 70% ethanol, dried and re-suspended in TE buffer (pH 8.0) with RNase. The final DNA concentration was estimated using a spectrophotometer.

3.3.4 Phenotypic screening

All the *Ds* lines have a *bar* gene that encodes for the phosphinothricin acetyl transferase (PAT) enzyme. This gene is involved in providing resistance to glufosinate containing herbicides (Singh et al., 2006; Bregitzer et al., 2007). This selection marker is crucial to screening for plants containing the *Ds* element after crossing with *Ac*TPase expressing plants (25-B). 0.2% of the herbicide IGNITE was prepared by mixing 50 µl of the herbicide in 25 ml of water. A young leaf from a plant at 3-4 leaf stage was chosen to perform this screening. Using a thick marker, the leaf was marked about 1 inch from the tip of the leaf and this area was treated with the herbicide solution using a cotton swab. The leaves were checked for necrosis 4-5 days after herbicide application. Healthy leaves indicated the presence of a *Ds* transposon. Leaves showed necrosis indicated the absence of *Ds* element, therefore, these plants were discarded. Further molecular analysis for detecting the occurrence of transposition of *Ds* is described below.

3.3.5 PCR analysis

To confirm the presence of the *Ds* element, a PCR amplifying the *Ds* construct was performed using the primers JNosF (5'-GCGCGGTGTCATCT-ATGTTACTAGATC-3') and JDs3R (5'-TATCCCGATCGAT TTCGAAC-3'). Plants containing original non-transposed *Ds* insertions were identified using primers that amplified a part of the *Ds* transposon and the flanking sequence of original *Ds* insertion site. The primers used for this purpose in the TNP-29 population are JlPF1 (5'-AACTAGCTCTACCGTTTCCG-3') and JPT29 3R (5'- TACGAACGCAC AAGTCACAC-3'). Primers used for the TNP-79 population were JIPF1 and JPT79 3R (5'- CTTCAGAGCAGTCGCATAGT-3'). To identify the presence of

*Ac*TPase, a PCR amplifying the *Ac*TPase construct was performed. The primers used for this purpose are the Ac3 (5'- ACCACCAGCACTGAACGCAGACTC-3') and Ac5 (5'- AACCTATTTGATGTTGAGGGATGC-3'). The positions of these primers are illustrated in Figures 3.2 and 3.3 below.

Each PCR reaction contained approximately 10 ng of genomic DNA, 1x PCR buffer (Promega), 200 µM of each dNTP, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 1% DMSO and 2.5U Taq DNA polymerase (Promega) in a 25 µl reaction. All PCRs were performed using a program consisting of an initial denaturation at 95°C for 2 min, followed by 36 cycles of 95°C denaturation step for 30 s, 60°C annealing step for 45 s and 72°C amplifying step for 60 s. PCR amplification was carried out using the GeneAmp PCR 9700 System (Applied Biosystems). Amplified products were analyzed by gel electrophoresis on 0.8% agarose gels.

Figure 3.2. Location of primers around the *Ds* construct for PCR analysis

Figure 3.3. Location of the *Ac* primers on the *Ac* construct for PCR analysis

3.3.6 DNA hybridization

DNA hybridization was performed using a chemiluminescence detection method. It consists of 3 major steps: preparation of the membrane, DIG labeling of the DNA probe and detection of the DIG labeled probe. Each step is described below.

3.3.6.1 Preparation of DNA membrane

Genomic DNA was obtained through the DNA extraction method described above. 5-20 µg of total genomic DNA was digested with *HindIII* restriction enzyme. Each digestion reaction contained 25U of *HindIII* and 1X reaction buffer in a total volume of 25 µl per reaction. The reaction was then

incubated at 37°C overnight to ensure complete digestion followed by a run using a 0.8% agarose gel at 30V overnight to ensure a good separation of genomic DNA fragments. The DNA was subsequently transferred onto a nylon membrane by a standard downward alkaline transfer method modified from Chomczynski (1992) and crossed linked to the membrane using a UV crosslinker and stored in a cool dry place for further use.

3.3.6.2 DIG labeling of DNA probe

DNA probe used for the detection of the *Ds* element was prepared using the PCR DIG-Probe Synthesis Kit (Roche). This PCR process integrates the digoxigenin (DIG) protein as a dUTP into the DNA sequence. The concentration of DIG-dUTP used is 1:3 DIG-dUTP:dTTP. Primers JNosF (5'-GCGCGGTGTC ATCTATGTTACTAGATC-3') and JEDs3 (5'-TGCTCACATGTTCTTTCCTGCG-3') were used to generate the probe. DNA template used to generate the probe was the pSP-*Ds*-Ubi-Bar construct described in Singh et al. (2006). The PCR cocktail contains 1X reaction buffer, 0.2 mM of each dATP, dGTP and dCTP, 130 µM dTTP, 70 µM DIG-dUTP, 0.5 µM of each primer, 2.6U of Taq polymerase and approximately 10 ng of template DNA in a 50 µl reaction. The PCR program involved an initial denaturation of the template DNA at 95°C for 2 min, followed by 36 cycles of denaturation, annealing and elongation steps at 95°C for 30 s, 60°C for 30 s and 72°C for 45 s, respectively. The reaction was finally held at 72°C for 5 min as the final elongation step. 5 µl of the PCR product was visualized on a 0.8% agarose gel to determine the successful integration of the DIG-dUTP and also to estimate the amount of probe for DNA hybridization.

PCR amplification was performed to determine the quantity of the DIG-labeled probe (Figure 3.4). By comparing the band size of the PCR products from 2) and 3) and also 4) and 5), an observation of an increase in the size of the DNA fragment signified the successful integration of the DIG protein into the DNA. Also the comparison of the band intensities between 2) and 4) and also 3) and 5) will determine the amount of DNA probe needed for DNA hybridization. As shown in Figure 3.4, the DIG was successfully integrated into the *Ds* probe and also the

intensity of the *Ds* probe was stronger than the intensity of the Roche control probe, therefore, 1.5 µl of the *Ds* probe per ml of hybridization buffer was used.

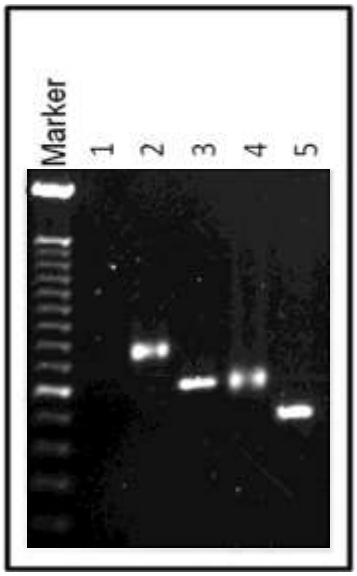

Figure 3.4. PCR product from DIG labeling of *Ds* probe. H_2O is used as a negative control. The PCR amplification product are indicated by the lane numbers. Lanes 1 to 5 are water, DIG labeled *Ds* probe, *Ds* probe without DIG labelling, Roche control with DIG label and Roche control without DIG labelling respectively.

3.3.6.3 DIG probe hybridization and detection

DNA membrane was prehybridized in a high-SDS hybridization buffer containing 50% deionized formamide, 7% SDS, 5X SSC, 0.1% N-lauroylsacrosine, 50 mM sodium phosphate buffer at pH 7.0 and 2% blocking reagent (Roche). This step was done for 90 min at the hybridizing temperature of 38°C before replacing the fresh hybridization buffer with the probe.

The hybridization temperature of the probe was calculated based on the formula: T_{hyb}= [16.6 log [Mol Na^+] + 0.41(%G + C) + 81.5] - 25°C - (0.72 x % formamide). The DIG-labeled probe was denatured by boiling for 5 min and placed on ice immediately. The probe was then mixed with pre-warmed High-SDS hybridization buffer and filtered using a polyethersulfonate (PES) filter of

0.45 µm pore size. The membrane was incubated in the solution containing the DIG labeled probe in the hybridization oven at the hybridizing temperature calculated at 38°C overnight. Next day, the solution containing the probe was removed and the membrane was washed twice with low stringency wash buffer (2X SSC and 1% SDS) and high stringency wash buffer (0.5X SSC and 0.1%), respectively. Subsequent detection steps were performed using the DIG Luminescent Detection Kit (Roche). The membrane was blocked with a 1 X blocking solution for 2 hours before incubating with a 1:10000 dilution DIG specific antibody conjugated to an alkaline phosphatase (AP) enzyme. The membrane was then washed four times for 10 min in washing buffer containing 100 mM maleic acid, 150 mM NaCl and 0.3% Tween 20 at the pH of 7.5. The pH of the membrane was equilibrated with a detection buffer containing 100 mM Tris-HCl and 100 mM NaCl (pH 9.5) before applying the chemiluminescent substrate disodium 3 -(4 -methoxyspiro {1,2 –dioxetane -3,2′ -(5′ -chloro) tricycle [3.3.1.1^{3,7}] decan} -4 -yl) phenyl phosphate) also known as CSPD. Finally, the membrane was incubated with the substrate for 10 min and exposed to an x-ray film.

3.3.7 Generation of flanking sequences

Flanking sequences were obtained with either HE-TAIL PCR or iPCR. The details of our newly developed HE-TAIL PCR are described in chapter 5. The iPCR technique is described below.

3.3.7.1 Nested iPCR

Genomic 5-10 µg of DNA from young leaf tissues of each new *Ds* insertion line was digested with *NcoI* or *NheI* restriction enzymes followed by heat inactivation. The total digested DNA was purified and self-ligated in a large reaction volume of 500 µl and 25U of T4 DNA ligase and was amplified by PCR with appropriate primers. The PCR cocktail was adjusted to a total volume of 50 µl per reaction, including 1X ExTaq DNA polymerase buffer, 0.2 mM dNTPs, 0.5 µM of each forward and reverse primers and ~10 ng of purified self-ligated genomic DNA. The PCR program involved heating at 94°C for 5 min before

adding 2.5U ExTaq DNA polymerase (TaKaRa). This was then followed by 36 cycles, each cycle consists of the denaturation, annealing and elongation steps at 94°C for 45 s, 60°C for 45 s, 72°C for 150 s, respectively (Cooper et al., 2004). Two sets of primers were used in this reaction to amplify 5' or 3' *Ds* end flanking sequence. For the 5' end, primers P3 (5'-GTAGATAATGCCAGCCTGTT-3') and P1 (5'-ATGTGCTACATTAAACTATG-3') or P3 (5'-CGACCGGATCGTATCGGT-3') were used. For the 3' end, primers P6 (5'-CATATTGCAGTCATCCGGAA-3') and P4 (5'-TGCGGAACGGCTAGAGCCAT-3') or P5 (5'-ACAGGTCGCATCCGTGTA CGAACG-3') were used. PCR products from the first PCR were diluted 20 - 50X with water and used as a template for the second PCR. The PCR for second round was performed with the same conditions except for the primers. Two sets of primers were used in this reaction for determining the 5' flanking sequence and the 3' flanking sequence. For the 5' end, primers P8 (5'-CTCGTGTTCTTGAGA CGCACACA-3') and P7 (5'-TTCGTTTCCGTCCCGCAAG T-3') were used. For the 3' end, primers P11 (5'-AACTAGCTCTACCGTTTCCG-3') and P10 (5'-TAGCAGCACGGATCTAACAC-3') were used. PCR products were subsequently run on a 0.8% agarose gel and bands were eluted using Gel extraction kit (Qiagen) and directly sequenced.

3.3.8 DNA Sequencing and basic bioinformatics analysis

Sequencing was performed at the McGill University and Genome Quebec Innovation Centre, (http://www.genomequebec.mcgill.ca/). These sequences generated from of iPCR and HE-TAIL PCR (described in chapter 5) were scanned for terminal inverted repeats (TIRs) and 8 bp duplication. Then, to identify putative genes that are significantly similar to that of the flanking sequence, expressed proteins and EST was found by using the various programs such as BLASTn, BLASTx and tBLASTx in NCBI (http://www.ncbi.nlm.nih.gov/). Sequences were then analyzed by bioinformatics analysis to determine the location of the *Ds* insertions in genic regions using programs such as GenScan (http://blast.wustl.edu/).

3.4. Results and discussions

3.4.1 Phenotypic screening

Analysis of a segregating F_2 population from the TNP-29 X 25-B (TNP-29A population) and TNP-79 X 25-B (TNP-79A population) crosses requires means to separating plants containing newly transposed *Ds* from all the other genetic combinations. For this purpose, herbicide containing 0.2% of the active compound, glufosinate ammonium was painted on leaves of three-week old plants. Due to the presence of the *bar* gene that encodes the phosphinothricin acetyl transferase (PAT) enzyme, plants containing the *Ds* construct are resistant to this herbicide (Figure 3.5). This is because the PAT enzyme can detoxify glufosinate ammonium by acetylating the amino group (Shin et al., 2008). Plants that are resistant to the herbicide were selected for subsequent molecular analysis.

However, it was observed that the efficiency of the phenotypic screening based on herbicide resistance was not very high. The low efficiency of the herbicide resistance can be explained due to unfavorable environmental conditions. It has been previously reported that the greater translocation of the herbicide in the leaf tissue under high humidity makes it more effective (Coetzer et al., 2001). Conversely, low humidity conditions can cause the plants to be more insensitive to this particular herbicide. The herbicide screening was only successfully applied to 93 plants out of 279 F_2 plants from a population (TNP-29A) created by crossing TNP-29 with *Ac*TPase expressing (25-B). Out of 93 plants, 48 were found to be resistant to the herbicide. This result was inconclusive to determine the presence of *Ds* due to the decreased sensitivity of the herbicide screening. Therefore, all plants were further screened by PCR analysis to reconfirm the presence of *Ds*.

Figure 3.5. Herbicide screening on barley palnts. The red lines indicate the portion of the leaf tip applied with herbicide. “R” indicates resistance and “S” indicates susceptability towards the herbicide

3.4.2 Molecular analysis

Molecular screening was performed on the F_2 population using PCR analysis in order to 1) identify the presence of the *Ds* element; 2) determine the transposition of *Ds* from its original insertion site; 3) determine the stability of the *Ds* lines based on the presence/absence of *Ac*.

A total of 279 plants from the TNP-29A population and 173 plants from the TNP-79A population were planted. DNA from each plant was extracted and screened by PCR using *Ds*-specific primers JNosF and JDs3R. Then, specific primers, one from *Ds* sequence, JIPF1 or JIPR5, and the other from the original flanking sequence, JPT29 3R or JPT79 5F, were used for identifying newly transposed *Ds* insertion lines in TNP-29 and -79, respectively. Amplification by these primers indicated that *Ds* did not transpose away from its original position. To identify a new *Ds* insertion, lines where *Ds* transposon amplification was

positive and amplification from the flanking sequence was negative were selected. Further, to separate stable and unstable *Ds* lines, the presence of the *Ac*TPase transposase was determined by PCR using primers Ac3 and Ac5. The lines containing *Ds* at a new position but without *Ac*TPase were considered as new stable *Ds* insertions and were used for further experimentation.

For all PCR experiments, positive and negative controls were used (Figure 3.6). Negative controls were water (H_2O) and Golden Promise (GP), a non transgenic (wild type) plant used for transformation. Other controls were the parent plants, TNP-29 or TNP-79. These lines served as a positive control for both the *Ds* and the flanking sequence PCR. The *Ds* plasmid, pSP-*Ds*-Ubibar-N, was also used as a positive control for *Ds*. Other controls include an *Ac* line, 25-B which is the plant containing the *Ac*TPase transposase, and the *Ac* plasmid, pUC-codA-Act1-*AcAc*, which acts as the positive controls to determine the presence of *Ac*TPase. Results from the PCR analysis are compiled in Table 3.1. The estimated band size for *Ds*, flanking sequence and *Ac*TPase PCR are 250 bp, 600 bp and 900 bp, respectively.

Based on the PCR analysis, new *Ds* insertions were calculated. As shown in Table 3.1, the frequencies of new *Ds* transpositions were 10% and 11% for TNP-29 and -79 populations, respectively. These results concur with previous publications regarding the *Ds* reinsertion frequencies which range from 11.8% to 17.1% in barley (Singh et al., 2006; Koprek et al., 2000). PCR screening is the preliminary step to determine new *Ds* transposition, for further reconfirmation of new *Ds* transposition events, Southern blotting was also performed.

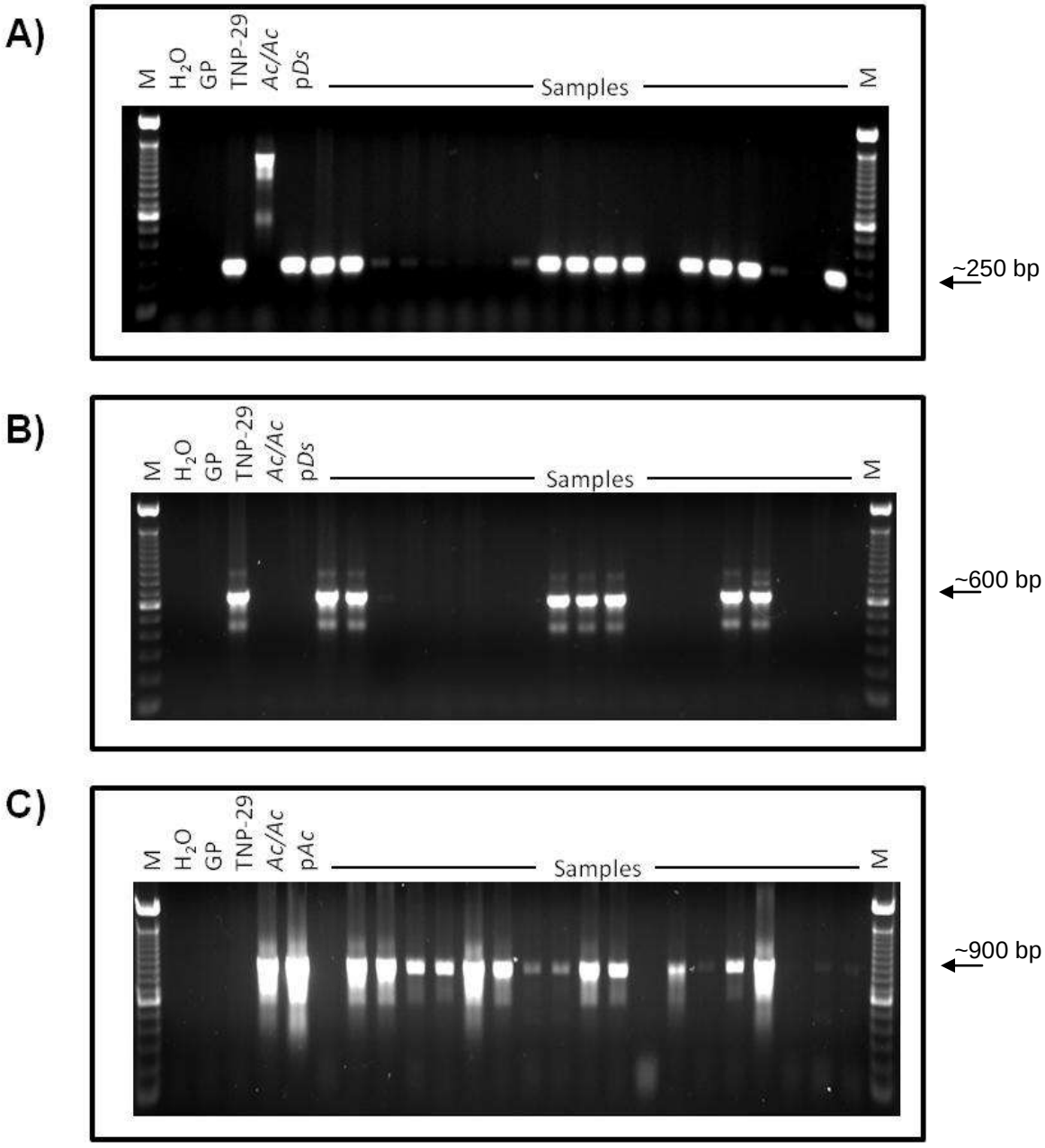

Figure 3.6. Gel pictures for PCR analysis for the TNP-29 population. "M" indicates the DNA ladder on all gels. Water and GP are used as the negative controls. TNP-29, *Ac/Ac* are plants that contain a single *Ds* insertion and *Ac*TPase, respectively. p*Ds* and p*Ac* are plasmids containing the *Ds* constrcut and *Ac* construct respectively. Arrows indicate the right band size of each PCR amplification. A) *Ds* PCR amplification indicating the presence of *Ds*. B) parental *Ds* PCR amplification indicating the original site of insertion. C) *Ac* PCR amplification indicating the presence of *Ac*TPase.

Table 3.1. Summary of *Ds* reactivation frequency from TNP-29 and -79 lines.

Population	TNP-29A	TNP-79A
Total number of plants screened	279	173
Total New *Ds* Transposition	28	19
Stable New *Ds* Transposition	18	11
Unstable New *Ds* (with Transposase)	10	8
Reactivation Frequency	10%	11%

3.4.3 DNA hybridization

Southern blotting was conducted for two major purposes: 1) to determine the number of *Ds* transposon copies present in the genome and 2) to verify the new *Ds* insertion, by comparing with the original *Ds* insertion line TNP-29. Figure 3.7 illustrates the results obtained from the Southern blotting analysis.

Controls for Southern blotting include a negative control (lane 1) of a non-transgenic parent plant, Golden Promise (GP), and a positive control (lane 2) which is the original parent plant with a single *Ds* insertion (TNP-29). Due to the absence of the *Ds* transposon in the non-transgenic GP, no bands were observed for this sample. However, the *Ds* probe hybridized with the genomic DNA from TNP-29, which produced a fragment of approximately 6 kb. This band was used as the reference band to determine the new transposition events. The samples used for Southern blotting analysis were selected based on the PCR results generated above. Observed bands through Southern blotting analysis were comapred to the 6 kb reference band from TNP-29. As shown in Figure 3.7, lanes 3 to 10 indicate samples with new transpositions. Band sizes different from 6 kb indicate new *Ds* transpositions. The number of observed bands per sample indicates the copy number of *Ds* transposon in a line.

As shown in Figure 3.7, bands present in lanes 3 to 10 indicate that these samples have new transpositions due to the different band sizes compared with the original TNP-29 insertion. However, the presence of 2 bands on lane 6 indicates that there are 2 copies of *Ds* transposon present in different locations in the genome of this particular sample. Seggregation of these transposons in subsequent generations will create new single copy *Ds* insertion lines. Many other faint bands were also observed in the Southern blotting (indicated by dotted arrows). These bands may be due to somatic nature of the *Ds* insertion happened only in few somatic cells. In order to further confirm these new *Ds* insertion lines, flanking sequences were obtained through iPCR described below.

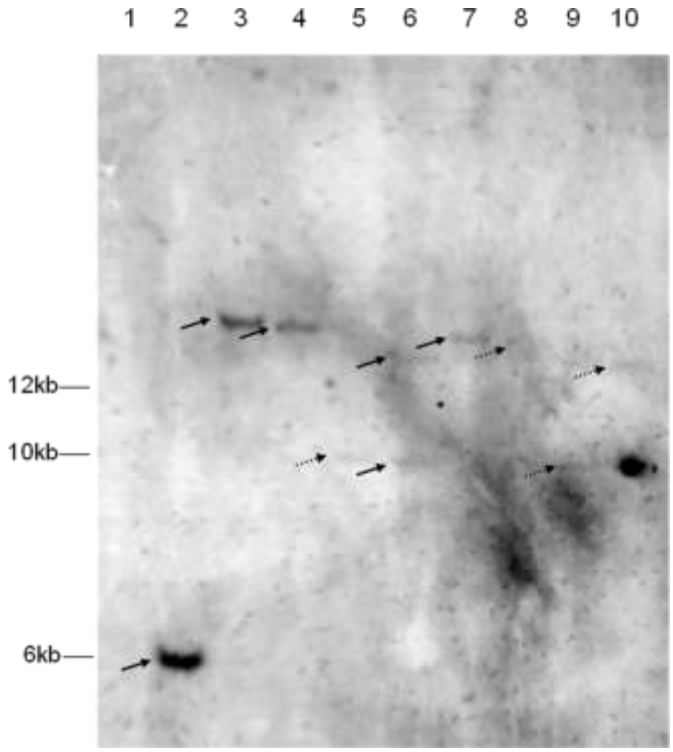

Figure 3.7. Southern blotting of digested genomic DNA for identifcation of new *Ds* transpositions. Lane 1: Golden promise (GP), a non-transgenic parent plant used as a negative control. Lane 2: TNP-29, the original *Ds* insertion line containing a single *Ds* insertion used as a positive contol and the reference size. Lanes 3 to 10: samples with new *Ds* transposition obtained from the F_2 population (TNP-29A).

3.4.4 Nested inverse PCR (iPCR)

It is known that the size of the genome inversely correleates to the effectiveness of PCR (Garner, 2002). In addition, due to the complexities of large genome, many PCR methods for obtaining flanking sequences are difficult to perform in large genome cereals. However, iPCR has been frequently used in crop plants to obtain flanking sequences because purification steps allow to increase chances of obtaining amplified flanking sequences.

iPCR was performed using two specific sets of nested *Ds* primers located close to the restriction sites of *NcoI* or *NheI* at the 5’ or 3’ end of the *Ds*

transposon. Amplified products contain fragments of DNA sequences from the flanking region of the *Ds* transposon. This method produced clean and abundant amplification products (Figure 3.8). Amplified products were observed in the first round of PCR amplification. PCR products with or without observable amplification during the first PCR cycle were further diluted before the application to another round of PCR amplification using nested primers. Each round of PCR cycle further increased the amount of amplification from the prceeding cycle. Several amplified bands in the same sample were individually isolated and extracted for seuqencing. As iPCR specifically amplifies flanking sequences with a specific band size, the presence of these bands indicates two possibilities. The first possibility can be explained by the secondary structures of the amplified flanking sequences. Another explanation for the presence of multiple bands may be the presence of two or more copies of *Ds* insertions in the sample. In order to confirm the reasons for the multiple bands, individual bands were extracted and visualized by gel electrophoresis. Single bands are the flanking seuqneces generated from the *Ds* insertion site. At the same time, multiple bands were also observed. These multiple bands were confirmed to be secondary structures produced during PCR amplification of the flanking sequences.

Although iPCR analysis provides clear amplification of flanking sequences, it is very tedious and laborious, involving many steps over a period of 3-5 days. In addition, iPCR needs many different enzymes which dramatically increase the cost. Due to the drawbacks of iPCR, a time-saving and cost-effetive method was deviced to generate more flanking sequences. This method was named as the High effiecincy thermal assymetric interlaced PCR (He-TAIL PCR) (Tan and Singh, 2011), the development of this aproach is specifically discussed in chapter 5.

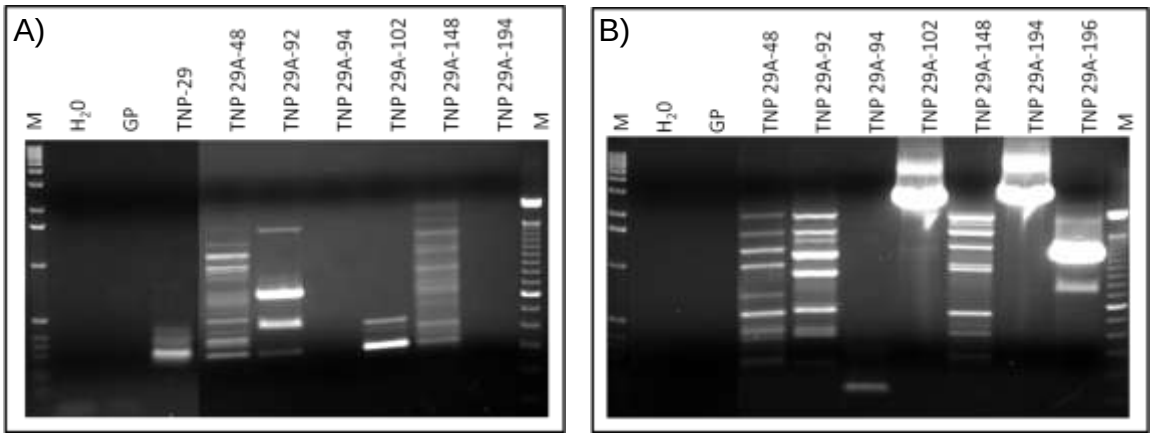

Figure 3.8. iPCR analysis of *Ds* insertion sites. The results above illustrate the iPCR amplification for the generation of flanking sequences from the *Ds* insertion sites. “M” indicates the 1 kb and 100 bp DNA ladder located on the left and right sides of each gel; Water, H_20, and Golden Promise, GP, are used as negative controls; “GP” is the non-transgenic plant; numbered lanes indicate samples containing new *Ds* insertions. A) and B) illustrate the second round of iPCR amplification at the 5’ and 3’ end of the *Ds* insertion respectively.

3.4.5 Basic bioinformatics analysis

Flanking sequences obtained from iPCR were used for bioinformatics analysis. The bioinformatics analysis provided information about the terminal inverted repeats (TIRs), 8 bp duplications, and the putative functions of these sequences. These results are compiled in Table 3.2. The information from the 5’ and 3’ flanking sequences enabled the determination of prefect and imperfect terminal invered repeats (TIRs) of the *Ds* transposon. These sequences are TTTCATCCCTA and TTTCATCCCTG for the 5’ and 3’ sides respectively. The imperfect TIRs, such as sample 2, 5, 7 and 10, may lead to the inability of the *Ds* reactivation in the subsequent generations (Singh et al., 2006).

As expected, 8 bp duplications, were produced during the *Ds* insertion (Pereira, 1998). In order to produce a contig of the 5’ and 3’ flanking sequences of the *Ds* insertion sites, these 8 bp duplicates greatly facilitate the matching of these sequences. This was to ensure that the sequences belong to the same *Ds*

insertion. Sequences were then applied to basic local alignment search tool (BLAST) analysis at NCBI (http://www.ncbi.nlm.nih.gov/) and GENE Scan (http://blast.wustl.edu/). Three major BLAST analysis were performed on the obtained sequences by the following softwares: BLASTn, BLASTx, and tBLASTx. BLASTn was used to identify neucleotide sequences that share very close neucleotide similarity to the flanking sequences generated from the *Ds* insertion lines. This analysis often does not produce results with significant e-values because the barley genome has not been sequenced. To obtain more information, the neucleotide sequence from the flanking sequences were translated into 6 amino acid sequences and queried against the protein database using BLASTx. Results obtained from the BLASTx analsysis have more significant e-values compared to the BLASTn analysis. tBLASTx analysis was performed if there is no sequence similarity observed from the BLASTn and BLASTx analysis. This program translated the neucleic acid sequence from the flanking sequences and all neucleotide sequences in the GENBANK database into 6 different amino acids sequences. The flanking sequences were then queried against the translated neucleotide database. Like BLASTx, tBLASTx produced more sequence similarity between the flanking sequences and the GENBANK database. This is because the codon redundancy of amino acids provides more combinations of translated neucleotide sequences. The sequences that did not match any of the BLAST analysis were further queried in the GenScan database.This program allows the discovery of putative exon regions that indicate the presence of a potential gene.

From the basic bioinformatic analysis of the newly genrated flanking sequences, six putative genes were identified. Although the relationship between these genes with malting quality still needs to be investigated, this study clearly indentified a *Ds* insertion in one known gene, the β-amylase. This finding concurs with the involvement of sugar degradation as one of the important malting quality parameter during the malting process (Swanston et al., 2002). Since the basic bioinformatic analysis only suggests the putative gene functions, to further confirm the gene functions, subsequent analysis such as reverse genetics remains to be done.

Table 3.2. Flanking sequences analysis for new TNP lines. For each flanking sequence, details for putative genes identified, e-values, status of each terminal inverted repeats (TIRs) and 8bp duplicates are indicated.

	Sample name	Side	Details	e-value	TIRs	8 bp duplicates
1	JA 112-33-62	5'	Unknown gene	N/A	TATCATCCCTA	ATTCCACC
2	JA112-33-62	3'	Unknown gene	N/A	TTTCATCCCCT	TCTAGTGG
3	TNP-29A-37	3'	Triticum aestivum cDNA, clone: WT008_L18	1E-104	TTTCATCCCTG	TATGGCAG
4	TNP-29A-50	5'	MDR-like ABC transporter	1E-18	TTTCATCCCTA	GTCGTGGC
5	TNP-29A-50	3'	MDR-like ABC transporter	2.1E-61	TTTCA	CAATGGTG
6	TNP-29A-92a	5'	POT Family protein	5E-39	TTTCATCCCTA	GTTTCAAA
7	TNP-29A-92b	3'	Glutamine Synthase	2E-55	TTTATCCCTA	GTAGGTGC
8	TNP-29A-94	3’	Unknown gene	N/A	TTTCATCCCTG	CCCCCACC
9	TNP-29A-194	5'	β-amylase	1E-20	TTTCATCCCTA	GTCGTGGC
10	TNP-29A-194	3'	MDR-like ABC transporter	2.9E-61	TTTCATG	GTGGTCCG
11	TNP-29A-208	5'	Unknown gene	N/A	TTTCATCCCTA	CTCCTGGG
12	TNP-29A-208	3'	Hordeum vulgare subsp. vulgare clone BAC 673I14	6E-61	TTTCATCCCTG	CCCCCACC

3.5 Conclusions

This study clearly demonstrates that the *Ac/Ds* transposon system can be effectively used in barley to tag genes. As proposed, the study successfully accomplished its objectives including identification of new transposition events, determination of the *Ds* reactivation frequency in TNP-29 and TNP-79; generation of *Ds* flanking sequences; and performance of basic bioinformatics of flanking sequences to discern putative gene function. These data generated will allow for the initiation of further investigations to saturate the malting quality QTL2. A total of 47 new transposition events were identified and the reactivation frequency of TNP-29 and -79 was determined to be in the expected range of 10-11%. Flanking sequences from 6 new *Ds* insertions were successfully obtained using nested inverse PCR (iPCR). Basic bioinformatics analysis indicates that *Ds* has preference to re-insert into genic regions. The identification of malting quality related genes (β-amlysae) suggests that the reactivation of *Ds* has great potential to identify malting quality related genes. Future directions of this study include development of additional TNP lines, generation of flanking sequences and genetic mapping of *Ds* loci. This effort will facilitate the saturation mutagenesis of the malting quality QTLs located on chromosome 4H to understand the effects of genes presnt in these QTLs in brewing and malting and to assist the development of functional markers in breeding programs for better barley malting varieties.

Connecting Statement

The main purpose of my thesis is to generate new *Ds* insertion lines to saturate the malting quality QTLs located on chromosome 4H. As described in Chapter 3, the *Ds* transposon was reactivated through conventional breeding methods by crossing single *Ds* insertion lines – TNP-29 and -79 with an *Ac*TPase expressing line – 25-B. However, this method is laborious and time consuming. In addition, partial or complete silencing of the *Ac*TPase in advance generations hampered further activation of *Ds* through cross breeding methods.

In this study (Chapter 4), we have devised an *in vitro* method for the reactivation of the *Ds* transposon through extra-chromosomal expression of *Ac*TPase. The success of this method will enable us to generate new *Ds* insertion lines more efficiently compared to the conventional breeding strategies.

I have contributed to all the work described in Chapter 4. The results from this chapter will be submitted to a relevant journal after reconfirmation of reactivation frequencies using other molecular approaches including Southern blotting and reverse transcription PCR (RT-PCR). The experiments performed in this study include the generation of the pCambia-pAct*GFP*-pUbi*Ac* construct, genetic transformation using *Agrobacterium*, the tissue culture of the transfected immature embryos and PCR based molecular screening for the regenerated plants. Funding for this project was provided by Dr. Jaswinder Singh through BMBRI and NSERC-CRD.

Chapter 4

Extra-chromosomal expression of transposase (*Ac*TPase) and reactivation of *Ds* transposons

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4.1. Abstract

Since the discovery of the *Activator* (*Ac*) and *Dissociation* (*Ds*) transposons by Barbara McClintok (1941), transposons have been widely used in plant genetic and genomic studies. Barley (*Horduem vulgare*) is the first *triticae* crop in which the maize *Ac/Ds* transposon system was exploited. In a two component *Ac/Ds* system, where *Ds* requires *Ac*TPase experession for its movements, the *Ac*TPase has been found to be silenced after few cycles of reactivation. Therefore, there is an urgent need to develop a new approach for continuous and efficient reactivation of *Ds* transposon. In this study, *Ds* transposon was reactivated through *in vitro* extra-chromosomal expression of *Ac*TPase. Immature barley embryos from three barley *Ds* insertion lines, TNP-13, -29 and -79, were transfected through *Agrobacterium* with a binary construct containing both the *Ac*TPase and the green fluorescent protein (*GFP*) genes. Tissues transiently expressing GFP were analyzed to monitor *Ds* movement. Screening of 544 transformed immature embryos indicates the successful reactivation of *Ds* transposon *in vitro* with high frequencies ranging between 10-30%. This frequency is significantly higher than the cross breeding approaches. More interestingly, reactivation of *Ds* transposon from a TNP line was observed at a frequency of 10.5% which, seldom happens with conventional methods. The successful utilization of this novel method would immensely facilitate efforts towards developing efficient functional genomics tools in barley.

4.2. Introduction

The reactivation of *Ds* transposons has been traditionally done by crossing of *Ds* insertion lines with *Ac*TPase expressing lines. Individual *Ds* and *Ac*TPase lines were generated via stable genetic transformation of immature barley embryos (Koprek et al., 2000). Since particle gun bombardment was used to develop the *Ac*TPase lines (McElroy et al., 1997), multiple copies of the *Ac*TPase gene have been observed in these lines. The presence of large copy

numbers of transgenes has been reported to induce transgene silencing (Meyer, 1998). Generally, genome wide silencing of the *Ac/Ds* transposons has been observed in maize (Brettell and Dennis, 1991). In heterologous species, where *Ac/Ds* system was introduced through genetic transformation, the decline in *Ds* transposition frequencies have also been correlated with reduced expression of *Ac*TPase. Studies in rice confirmed the loss of or reduced *Ds* activity during generation advance due to a pronounced reduction of *Ac*TPase expression by epigenetic silencing (Upadhyaya et al., 2002; Nakagawa et al., 2000). Transposition were rarely seen in F_3, F_4 generations due to *Ds* inactivation and loss of *Ac*TPase activity (Chin et al., 1999; Greco et al., 2003). Similarly, reduced activity or complete silencing of the *Ac*TPase transposase was observed in barley at the T_3 and subsequent generations (Koprek et al., 2000). In order to maintain the *Ac*Tpase expression, generation advancement of available *Ac*Tpase lines has been avoided. Consequently, constant development of new transgenic *Ac*TPase lines is always required in order to reactivate *Ds* from TNP lines through cross breeding. However, in barley, the development of new *Ac*TPase lines through genetic transformation is cumbersome. Therefore, it is necessary to develop alternative sources of transposase for the reactivation of *Ds* transposons.

Using a modified *Agrobacterium* transformation protocol, we devised an *in vitro* extra-chromosomal method to transiently express the *Ac*TPase in immature barley embryos. Although the extra-chromosomal expression method has not been extensively studied, transient expression of an extra-chromosomal gene was shown to be successful in tobacco gene silencing studies (English et al., 1997). Recently, Upadhyaya et al. (2006) have reported reactivation of *Ds* in rice through the transient infection with *Agrobacterium* containing immobile *Ac* construct. Despite the limited reports in plant science literature regarding extra-chromosomal expression of a transposase, this system has been extensively used in *Drosophila*. A *Drosophila* transposon – P element, was introduced into the *Drosophila* genome through extra-chromosomal expression of the transposase (Karess and Rubin, 1984). This method has been routinely used to introduce transgenes into the *Drosophila* genome.

Based on the previous successes, we investigated the potential of transient extra-chromosomal activity of transposase in immature barley embryos to activate *Ds*. For this purpose, we selected the same candidate barley *Ds* lines which were reactivated previously through conventional approaches. These lines are TNP-29 and -79, located near the malting quality QTLs on chromosome 4H. Selection of these lines also allowed us to achieve our objectives to saturate the malting quality QTLs on chromosome 4H with *Ac*/*Ds* transposons. The *Ds* transposons were reactivated in immature barley embryos from these lines through the introduction of a cassette carrying the *Ac*TPase and GFP genes. New *Ds* insertion lines generated from this study can contribute to the search of gene/genes located in the malting quality QTL of barley.

4.3. Materials and Method

The general overview of experimental method is shown in Figure 4.1.

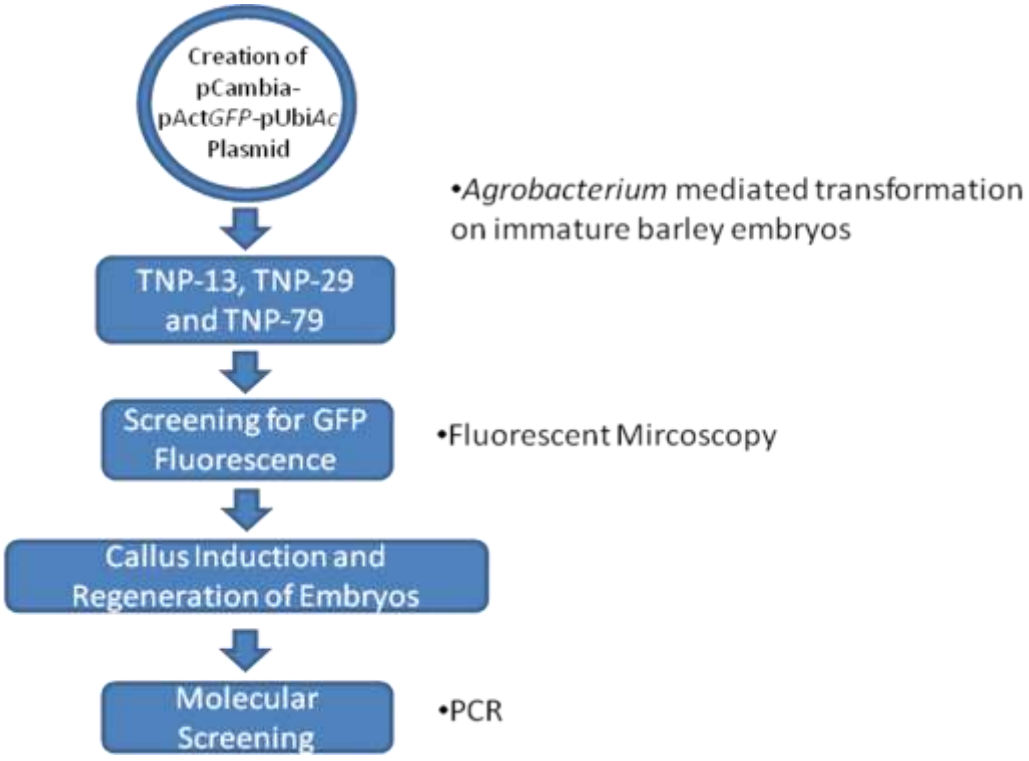


Figure 4.1. Strategy for the transient expression of *Ac*TPase and the development of new TNPs.

4.3.1. Generation of pCambia-pAct*GFP*-pUbi*Ac* construct

The construction of pCambia-pAct*GFP*-pUbi*Ac* construct was carried out in two steps. This construct contains an *Ac*TPase gene under the control of a Ubiquitin promoter (pUbi*Ac*), and a *GFP* gene driven by an actin promoter (pAct*GFP*) in a pCambia 1300 binary vector. Both the *Ac*TPase and *GFP* genes were fused to a nopaline synthase (nos) terminator. Plasmids containing the *Ac*TPase and *GFP* genes were previously constructed (Koprek et al., 2000; Cho et al., 2003; Singh, unpublished). The pCambia 1300 vector is a commercial binary vector suitable for *Agrobacterium tumefaciens*. It contains the kanamycin and hygromycin antibiotic resistance genes which are selection markers for the plasmid in bacteria and plant cells, respectively. It also contains a multiple cloning site (MCS) which allows for the insertion of DNA fragments into the region between the left and right borders of the Ti plasmid. The genes located within the left and right borders can be transferred into the host cell by *Agrobacterium* during the infection.

4.3.1.1. Plasmid construction

The pUbi*Ac* containing plasmid was digested with the *Pst*I restriction enzyme in a 25 µl reaction. This reaction contained 2 µg of plasmid DNA, 5U of *Pst*I and 1X reaction buffer and was incubated at 37°C overnight. A 6.4 kb pUbi*Ac* cassette was purified and ligated into the pCambia 1300 binary vector at the *Pst*I site.

After the construction of pCambia-Ubi*Ac*, it was digested with *Bam*HI and *Kpn*I at the multiple cloning site (MCS). The 25 µl restriction digest reaction contained 2 µg of plasmid DNA, 5U of each restriction enzyme – *BamH*I and *Kpn*I, and 1X reaction buffer and was incubated at 37°C overnight.

Another plasmid containing the *GFP* gene was completely digested with *Kpn*I before applying to a partial digestion using the *Bam*HI restriction enzyme. The 25 µl digestion reaction contained 5 µg of plasmid DNA, 5U of *Kpn*I and 1X reaction buffer and was incubated at 37°C overnight to ensure a complete digestion. Then the reaction was applied to a partial digestion. The reaction from

the complete digestion was added with 1 µl of 10X diluted *Bam*HI enzyme. This reaction was incubated at 37°C for only 15 min. The 15.3 kb pCambia-pUbi*Ac* fragment and 2.4 kb pAct*GFP* cassette were then purified by gel extraction followed by ligation.

Following the ligation, the plasmids were transformed into competent DH5α *E.coli* cells through heat shock treatment. The total ligation reaction was mixed with 200 µl of competent *E.coli* cells, incubated at 4°C for 10 min before applying heat shock at 42°C for 90 s. The microcentrifuge tube containing the mixture was incubated on ice for another 30 min before adding 800 µl of Lysogeny Broth (LB) media into the mixture. The microcentrifuge tube was then incubated on a shaker at 230 rpm at 37°C for another 60 min before being plated on LB media containing 50 mg/L Kanamycin. Then, bacterial colonies growing on the selection media were cultured, and the constructs were purified using DNA miniprep (Qiagen, Valencia, California) and verified using restriction enzymes, *Eco*RI, *Xho*I and *Nco*I.

4.4.2. Transformation of *Agrobacterium* strain AGL1

Heat shock competent AGL1 *Agrobacterium* cells were prepared by growing the bacteria in the YEP media within a shaker at 180 rpm at 28°C overnight. 2 ml of this culture was added into 50 ml of yeast extract-peptone (YEP) media and then incubated in a shaker at 180 rpm at 28°C until the O.D. falls within the range of 0.5 to 1.0. The cell culture was then centrifuged at 3000 g at 4°C for 5 min before re-suspending in 1 ml of 20 mM $CaCl_2$. 100 µl of *Agrobacterium* suspension was then allocated in separate microcentrifuge tubes and immediately frozen.

The newly constructed pCambia-pAct*GFP*-pUbi*Ac* plasmid was introduced into competent cells of AGL1 by heat shock transformation. The 20 ng of purified plasmid was mixed into a briefly thawed culture of 200 µl competent AGL1. The micro-centrifuge tube containing the *Agrobacterium* and the pCambia-pAct*GFP*-pUbi*Ac* plasmid was immediately placed into liquid nitrogen and then thawed at 37°C for 5 min. The reaction was mixed with 800 µl of YEP media and

cells were grown at 28°C on a shaker at 180 rpm for 2.5 hours. Subsequently, the cell culture was plated on a YEP media containing 50 mg/L Kanamycin to select for transformed colonies.

4.3.3. Preparation of bacterial culture for *Agrobacterium*

The transformed *Agrobacterium* was grown in 50 ml of MG/L media containing 100 mg/L Kanamycin in a shaker at 180 rpm at 27°C until the O.D. reaches 0.4 at the wavelength of 550 nm. A working stock of bacterial culture was prepared by adding 200 µl of 15% glycerol into 200 µl of bacterial culture. This culture was then kept at room temperature for 6 hours before transferring to -80°C for long term storage (Tingay et al., 1997). A day before transformation, one tube of working stock was thawed and mixed with 9.6 ml of MG/L media containing 100 mg/L Kanamycin and then incubated in a shaker at 180 rpm at 28°C overnight. The full strength bacterial inoculum having an O.D. of 0.4 was used for transformation. Approximately 30 embryos were infected using 1 ml of *Agrobacterium* culture per transformation event. *Agrobacterium* culture used for transformation was prepared by spinning the culture at 5000 rpm for 5 min, followed by the removal of liquid MG/L media before re-suspending the bacterial pallet in 1 ml of co-cultivation (CO) media.

4.3.4. Preparation of barley immature embryos

Three barley lines, TNP-13, -29 and -79 were planted in the growth chamber. These lines were selected based on their heterozygosity and status of the terminal inverted repeats (TIRs). TNP-29 is a heterozygous line and TNP-79 is a homozygous line for the *Ds* insertion, both of them have intact TIRs. TNP-13 is a heterozygougs line with a defective TIR. These plants were planted in the growth chamber using conditions mentioned in Section 3.4.1 of Chapter 3.

Barley kernels at 10-14 days post antherisis stage were selected from each line. These kernels were surface sterilized using 70% ethanol for 5 min, and washed with 20% bleach for 20 min. Two washes with sterilized water were performed following each sterilization step. Immature embryos were then

removed from the kernels using a scalpel and a forceps under sterile conditions. Approximately 30 embryos were placed in a 1.5 ml microcentrifuge tube containing 1 ml of co-cultivation (CO) media for *Agrobacterium* transformation.

4.3.5. Infection of immature embryos with *Agrobacterium*

Excess co-cultivation (CO) media was removed from the microcentrifuge tube containing sterile barley immature embryos. These immature embryos were incubated in a water bath at 43°C for 3 min and then at 25°C for 2 min before introducing 1 ml of bacterial culture. The mixture was incubated at room temperature for 15 min. This protocol was shown to be optimal for *Agrobacterium* transformation of immature sorghum embryos (Gurel et al., 2009). After the incubation, the immature embryos were transferred onto a solid CO media to enhance *Agrobacterium* transformation. Then these embryos were transferred onto a callus induction (CI) media, with the scutellum placed on the media. The CI media containing 50 mg/L Hygromycin and 300 mg/L Timentin can eliminate untransformed tissue and *Agrobacterium* growth. The immature embryos were incubated in the dark on the CI media for further screening.

4.3.6. Detection of embryos with extra-chromosomal expression

At the 3rd, 10th and 17th days post inoculation (dpi), immature embryos were screened for GFP fluorescence. The fluorescence microscopy work was done by using a Nikon SMZ1500 stereomicroscope with a GFP-1 filter and an excitation wavelength of 480/40 nm, in the core facility at the Institute of Parasitology at McGill University. The images were captured by QIMAGING QICAM Fast 1394 monochrome camera. The embryos expressing GFP were separated from non-GFP expressing embryos and were sub-cultured onto to subsequent tissue culture media for regeneration as described below.

4.3.7. Regeneration of embryos

Embryos were regenerated into full grown plants by sub-culturing the calli on media modified from Bartlett et al. (2008). All tissue culture media

containing 300 mg/L Timentin were used to inhibit *Agrobacterium* growth. After 2 weeks of sub-culturing on CI media, the calli were transferred onto a rooting transition (RT) media and kept under a piece of paper for a reduced light intensity of 75 µmol/sm^2 with a photoperiod of 16/8 h light/dark for 2 weeks. This media allowed the regeneration of shoots from the barley calli. The regenerated shoots were then transferred onto the rooting (R) media prepared in Magenta boxes and kept at a full light intensity of 140 µmol/sm^2 with a photoperiod of 16/8 h light/ dark. The R media enabled the regeneration of roots before transferring the plants into soil. When significant amount of roots were regenerated, plantlets were uprooted and transferred into soil. These plantlets were covered with a transparent plastic lid for 2 days to maintain the humidity of approximately 70%. The transparent lid was removed 2 days later to expose the plants to ambient humidity.

4.3.8. Barley tissue culture media

All media used in this study were sterilized by autoclaving for 22 min. All antibiotics and hormones were sterilized by filter sterilization using 0.22 µm syringe filters of mixed cellulose ester (MCE) membranes. The Co-Cultivation media (CO) and the Callus Induction (CI) media contained 4.3 g/L Murashige & Skoog plant salt base, 30 g/L Maltose, 1.0 g/L Casein hydrolysate, 350 mg/L Myo-inositol, 690 mg/L Proline, 1.0 mg/L Thiamine HCl, 2.5 mg/L Dicamba and 3.5 g/L Phytagel. 1.25 mg/L $CuSO_4 \cdot 5H_2O$ and 50 mg/L Hygromycin were added only to the CI media. For the Rooting Transition (RT) and Rooting (R) media, 2.7 g/L Murashige & Skoog modified plant salt base (without NH_4NO_3), 20 g/L Maltose, 165 mg/L NH_4NO_3, 750 mg/L Glutamine, 100 mg/L Myo-inositol, 0.4 mg/L Thiamine HCl, and 3.5 g/L Phytagel were added. In addition, 1.25 mg/L, $CuSO_4 \cdot 5H_2O$, 2.5 mg/L 2, 4-Dichlorophenoxy acetic acid (2,4-D) and 0.1 mg/L 6-Benzylaminopurine (BAP) were added only to the RT media. All the above media except the CO media contain 300 mg/L Timentin.

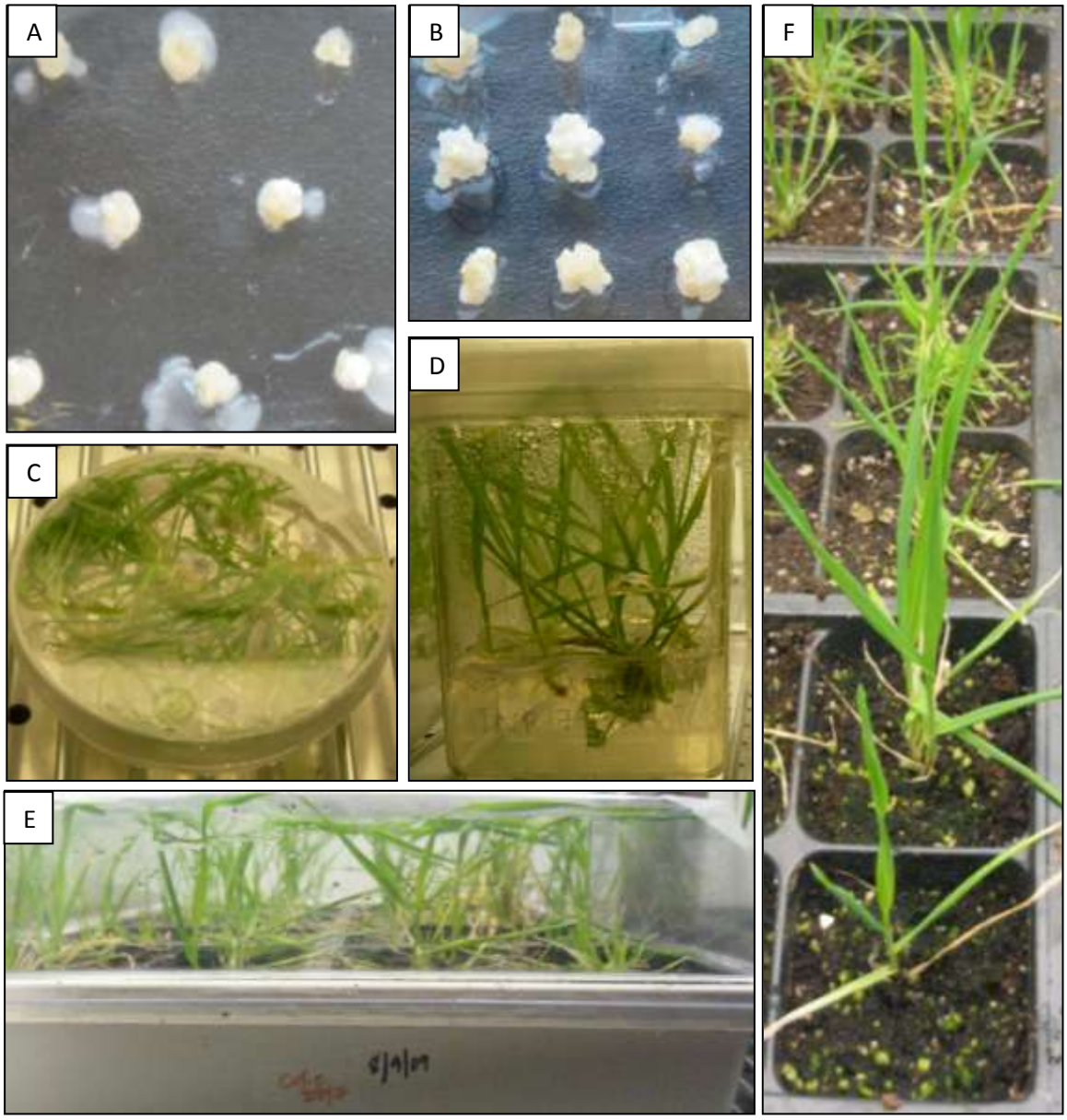


Figure 4.2. Tissue culture experiment process. A) Co-cultivation of immature barley embryos with *Agrobacterium tumefaciens* strain AGL1 on a co-cultivation media. B) Callus induction and selection of immature embryos on a callus induction media containing hygromycin as a selection pressure. C) Regeneration of shoots from the callus formed on a rooting transition media. D) Regeneration of roots in the regeneration media for all green shoots. E) Transfer of regenerated plantlets into soil with plastic lid. F) Normal growth of the regenerated plantlets in the growth chamber.

4.3.9. Molecular analysis of *Ds* transposition

Newly generated plants were screened for new *Ds* transpositions using PCR analysis. Four types of PCR analyses were conducted, including empty donor PCR, flanking sequence PCR, *Ds* PCR and *Ac* PCR.

The empty donor PCR was performed with primers in the flanking regions on both sides of the original insertion sites of *Ds* to determine the transposition of *Ds* away from its original insertion sites. For this purpose, three sets of primers were used, each specific for the three different *Ds* lines. These primers are JPT13 5R (5'-CCCTCCCGGTTTGTTTGTGT-3') with JPT13 3R (5'-GCACTCAGCCACAATGACAA-3'), JPT295R (5'-GGTCGACACCTCCACTGTAG -3') with JPT29 3R (5'-TA CGAACGCACAAGTCACAC-3') and JPT79 5R (5'-GGAGCCATGAGTAGGATTGT-3') with JPT79 3R (5'-CTTCAGAGCAGTCGCAT AGT-3') for TNP -13, -29 and -79, respectively.

The flanking sequence PCR was used to determine if the *Ds* transposon is excised during the transient expression of *Ac*TPase. This PCR aims to amplify a portion of the *Ds* transposon along with a part of the flanking sequence. Primers used for this purpose are JNosF (5'-GCGCGGTGTCATCTATGTTA CTAGATC-3') with JPT13 3R (5'-GCACTCAGCCACAATGACAA-3') or JPT29 3R (5'-TACGAACGCACAAGTCACAC-3') or JPT79 3R (5'-CTTCAGAGCAGTCGCA TAGT-3') for the TNP -13, -29 and -79, respectively.

The third one was *Ds* PCR. This PCR aims to amplify the *Ds* transposon using the primers JNosF (5'-GCGCGGTGTCATCTATGTTACTAG ATC-3') and JDs3R (5'-TATCCCGATCGATTTCGAAC-3').

Finally, the *Ac* PCR was performed to confirm the presence of *Ac*. This PCR aims to amplify the *Ac*TPase gene using primers Ac3 (5'-ACCACCAGCACTGAACGCAGACTC-3') and Ac5 (5'- AACCTATTTGATGTTGA GGGATGC-3') to determine if the *Ac*TPase was transfected into the immature barley embryos during *Agrobacterium* transformation.

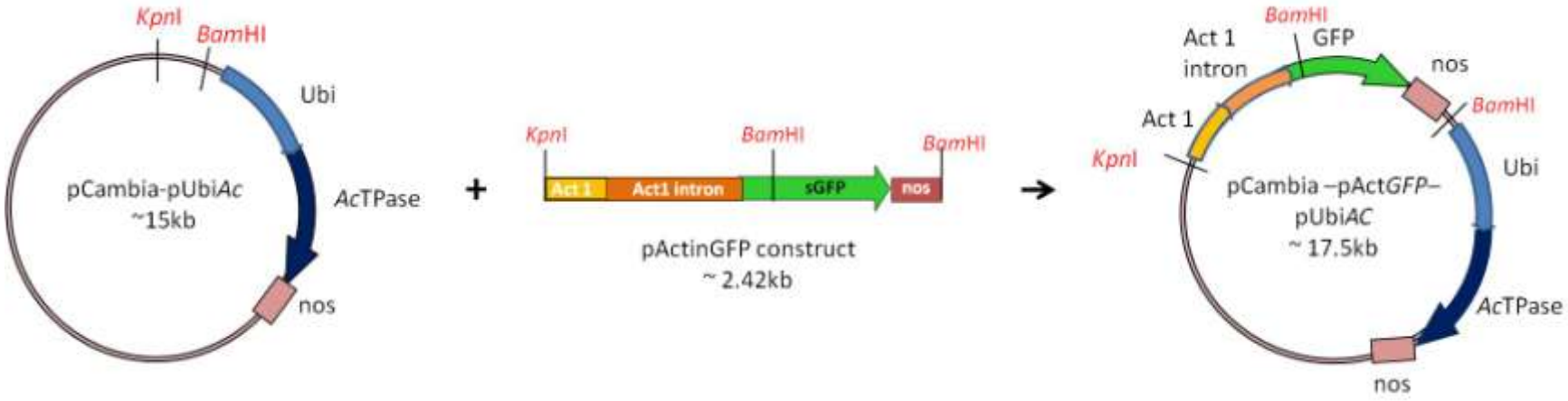
The locations of the described primers used in the empty donor PCR is illustrated in Figure 4.3. All other primers were illustrated in Figure 3.2 and 3.3 in Chapter 3. PCR was conducted as described in Section 3.3.5, Chapter 3.

Figure 4.3. Locations of the empty donor PCR primers used.

4.4. Results and discussion

4.4.1. Generation of the *GFP-Ac*TPase construct

In order to transiently express the *Ac*TPase genes in immature embryos to reactivate the *Ds* transposons, an appropriate construct was required. As *GFP* is commonly used a visual marker to monitor genetic transformation, it was linked with *Ac*TPase in a single construct. The binary construct generated for this purpose is illustrated in Figure 4.4. It contains both the *Ac*TPase and *GFP* genes within the left and right borders of the same plasmid. Prior to transformation, this construct was verified by restriction digestion analysis. Three different restriction enzymes – *Eco*R*I*, *Xho*I and *Nco*I, were chosen based on the restriction sites to confirm the newly generated construct (Figure 4.5). Samples used to perform the restriction digestion analysis include two newly generated constructs, pCambia-pAct*GFP*-pUbi*Ac*, lanes 1 and 2, and the original construct, pCambia-Ubi*Ac*, lane 3. The size of digested fragments was compared with the expected band sizes for these constructs (Table 4.1). The size of the DNA fragments obtained from this restriction analysis confirmed proper assembly of the pCambia-pAct*GFP*-pUbi*Ac* construct.



pCambia-pUbi*Ac* pAct*GFP* pCambia-pAct*GFP*-pUbi*Ac*

Figure 4.4. Construction of the pCambia-pAct*GFP*-pUbi*Ac* plasmid. The red restiction sites indicate the sites where the plasmid was digested and ligated with the *GFP* cassete.

Table 4.1. Expected band sizes for restriction digestion analysis.

	***Eco*RI**	***Xho*I**	***Nco*I**
pCambia-	0.4kb	1.0kb	2.0kb
pAct*GFP*-pUbi*Ac*	1.4kb	1.1kb	15.4kb
	1.7kb	3.2kb	
	1.9kb	4.0kb	
	13kb	8.1kb	
pCambia-pUbi*Ac*	1.4kb	1.1kb	15.4kb
	1.7kb	1.8kb	
	11.9kb	4.0kb	

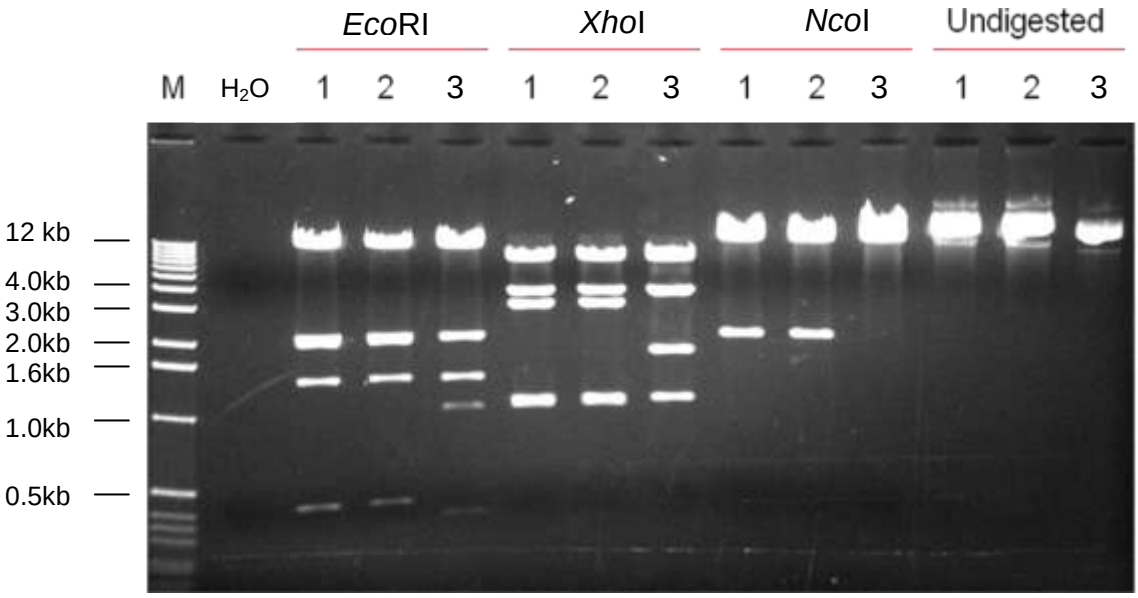


Figure 4.5. Restriction digestion analysis to determine the identity of the pCambia-pAct*GFP*-pUbiAc construct. "M" indicates the DNA ladder, *Eco*RI, *Xho*I and *Nco*I indicate restriction enzymes for the analysis. Lanes 1 and 2 correspond to the newly generated construct, pCambia-pAct*GFP*-pUbi*Ac*. Lane 3 corresponds to the construct pCambia-pUbiAc

4.4.2. Genetic transformation

The main goal of this experiment was to transiently express the *Ac*TPase genes in immature embryos to reactivate the *Ds* transposons. Hence, *Ac*TPase and *GFP* were linked and ubiquitously expressed in the immature embryos. GFP was used as a visual marker for the *Ac*TPase expression.

Using this new construct, *Agrobacterium*-mediated transformation was performed on immature embryos to transiently express *Ac*TPase. Because an antibiotic-resistant gene can confer resistance to hygromycin (hpt), selection of transformed tissues was done based on antibiotic resistance. In addition, the presence of the *GFP* gene allows the selection of tissues transiently expressing *Ac*TPase based on the GFP fluorescence as a visual marker.

A total of 544 embryos from 3 different TNP lines – TNP-13, -29 and -79 were used for the transformation. Embryos from these lines were used for super-transformation, a genetic transformation of already transformed tissues (Upadhyaya et al., 2006), using the pCambia-pAct*GFP*-pUbi*Ac* construct. Embryos expressing GFP were marked after each screening session. The frequency of transformed embryos expressing GFP fluorescence on the 3rd, 10th and 17th dpi was 49.6%, 16.7% and 7.9%, respectively. A total of 164 embryos were successfully regenerated into shoots. However, multiple shoots were regenerated for some embryos (Table 4.2), therefore, a total of 177 shoots were successfully regenerated into full grown plantlets. These shoots were then utilized for subsequent molecular analysis. The summary of the total regenerated shoots and plantlets were compiled in Table 4.2.

All embryos that underwent *Agrobacterium* transformation were regenerated into plants regardless of GFP expression. This step was to ensure the maximum number of *Ds* transpositions to be detected during the transient expression of *Ac*TPase. Initial transformations were subjected to 150 mg/L Timentin to inhibit *Agrobacterium* growth. Unfortunately, this concentration was too low. Hence, the antibiotics concentration was increased to 300 mg/L. This antibiotic concentration was used without adverse effects on the growth of callus. However, it has been reported that Timentin concentration greater than 300 mg/L

was found to cause the brownness of explants, thus affecting the embryogenesis and regeneration of the explants (Ministry of Agriculture, Fisheries and Food, 2000).

Also, in order to reduce the number of non-transfected tissues, all embryos were subjected to hygromycin selection at the concentration of 50 mg/L for 30 days post inoculation. This selection pressure imposed on the immature embryos is relatively low compared to other transformation protocols (Bartlett et al., 2006; Hensel et al., 2009). However, since our interest is to transiently express the *Ac*TPase in the immature barley embryos, stringent selection pressure was reduced to avoid stable transformation of the barley embryos.

Table 4.2. Summary of transformation of immature embryos and regeneration of transgenic plants. Successful embryos generated from barley single *Ds* insertion lines TNP-13, -29 and -79 at the end of each transformation stage are compiled in the table.

	Total Embryos transformed	**Total Embryos Emitting GFP fluorescence**	**Total embryos regenerated into shoots**	**Total plantlets regenerated**
TNP-13	179	100	47	57
TNP-29	143	68	49	46
TNP-79	222	102	68	74
Total	544	270	164	177

4.4.3. Screening for transient expression of *Ac*TPase

The green fluorescent protein (GFP) screening for inoculated embryos was done on the 3rd, 10th and 17th day post inoculation (dpi). The selected time was based on the existing literature regarding transient expression of the GFP. Transient expression of GFP was observed to be at its maximum from 1.5 to 3 days post co-cultivation (Dhillon et al., 2009; Upadhyaya et al., 2006). Approximately 50% of the total inoculated embryos exhibited GFP expression on the 3rd dpi. We have also observed a negative correlation between the GFP

expression and the number of days post inoculation. GFP expression decreased from a frequency of 49.6 % on the 3rd dpi to 7.9% on the 17th dpi. This data is in agreement with the definition of transient expression, which refers to the rapid increase and subsequent decline of gene expression in cells or tissues after the introduction of a transgene (Dhillon et al., 2009). We have also observed a positive correlation between the size of GFP spots in transfected tissues and the number of dpi (Figure 4.6). This expression pattern indicates the presence of stable *Ac*TPase transformation.

Although GFP has been used as a visual marker in many species, interfering wavelengths emitted due to secondary metabolites and chlorophyll can cause complications during GFP screening (Zhang et al., 2010). These interfering wavelengths are collectively known as auto-fluorescence. Plant tissues have been observed to emit auto-fluorescence of various wavelengths especially around damaged tissues, where secondary metabolites are produced in response to wounding. Auto-fluorescence can be filtered by different colors and also by narrowing the range of the emission filter for selecting GFP emission wavelength (Hily and Liu, 2008). However, due to the low intensity of GFP fluorescence emission, narrowing down the detectable wavelength range can increase the number of false negative tissues, thereby reducing the number of plants transiently expressing the *Ac*TPase.

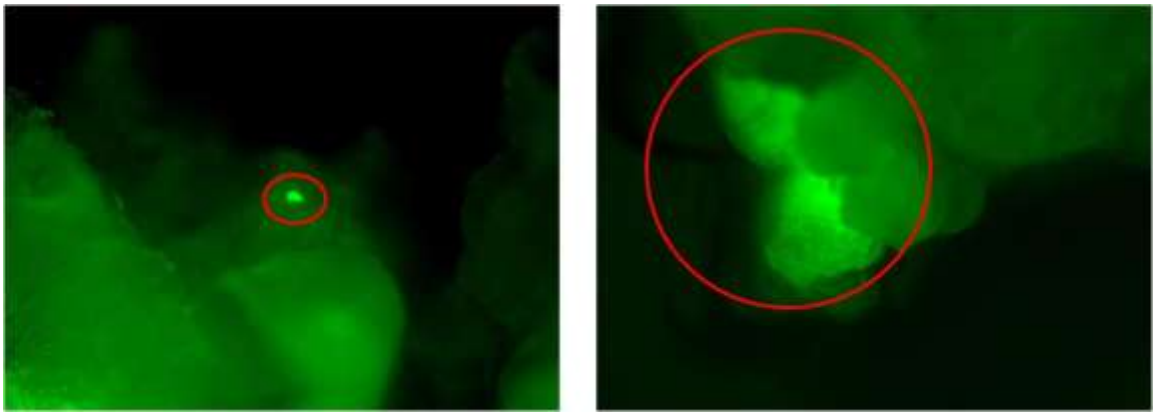


Figure 4.6. GFP fluorescence for tissue culture samples of TNP-79 on the 3rd (left) and 17th (right) dpi. The red circle indicates the detected GFP fluorescence.

4.4.5. Molecular analysis of regenerated plants

Molecular analyses were conducted for all 177 regenerated plantlets. These plants were categorized into GFP positive and negative plants. PCR analyses performed on the samples were based on 4 different PCR amplifications which are flanking sequence PCR, empty donor PCR, *Ds* PCR and *Ac* PCR. Due to the homozygous *Ds* insertions in TNP-79, plantlets with no amplification from the flanking sequence PCR were selected as putative lines with *Ds* transpositions. As for the heterozygous *Ds* insertion lines of TNP-13 and -29, plantlets with a negative flanking sequence PCR amplification and a positive empty donor PCR amplification were selected for further verification of the presence of *Ds* transposon.

For the empty donor PCR analysis illustrated in Figure 4.7 A, the presence of empty donor PCR amplification without a *Ds* insertion was indicated by a smaller band size on the gel (Figure 4.7 A, lane “GP”). If the *Ds* is present within the amplified region, the amplicon will be approximately 3.6 kb larger than the smaller amplified fragment (Figure 4.7 A, lane “parent line”). Samples containing both band sizes indicate that there is only one *Ds* insertion in that particular sample. This analysis is especially useful for the analysis of lines generated from the homozygous TNP-79 line. The presence of either the small PCR amplicon or both band sizes in a particular sample will indicate that a *Ds* transposition occurred. However, based on the PCR analyses, no samples indicated the transposition of both *Ds* transposon copies in TNP-79. This observation can be explained by the absence of *Ac*TPase in the tissue. Alternatively, heavy methylation patterns at the terminal repeats of the *Ac*/*Ds* transposons could also inhibit the transposition of the *Ac*/*Ds* transposons on either strand of the DNA (Wang et al., 1996).

Amplification of the *Ac*TPase gene was observed in one of the samples (Figure 4.6 C), although this band was much fainter than the positive control. As the positive control used in this PCR is an *Ac*TPase expressing plant generated through particle gun bombardment (Lemaux et al., 1996), there are multiple copies of the *Ac*TPase gene present in this control. Studies have indicated that

transgene number is much higher in transgenic plants produced through biolistic methods than through *Agrobacterium* mediated transformation (Cheng et al., 2001). The faint amplification in one sample may be explained by the number of copies of the *Ac*TPase gene present in the genome.

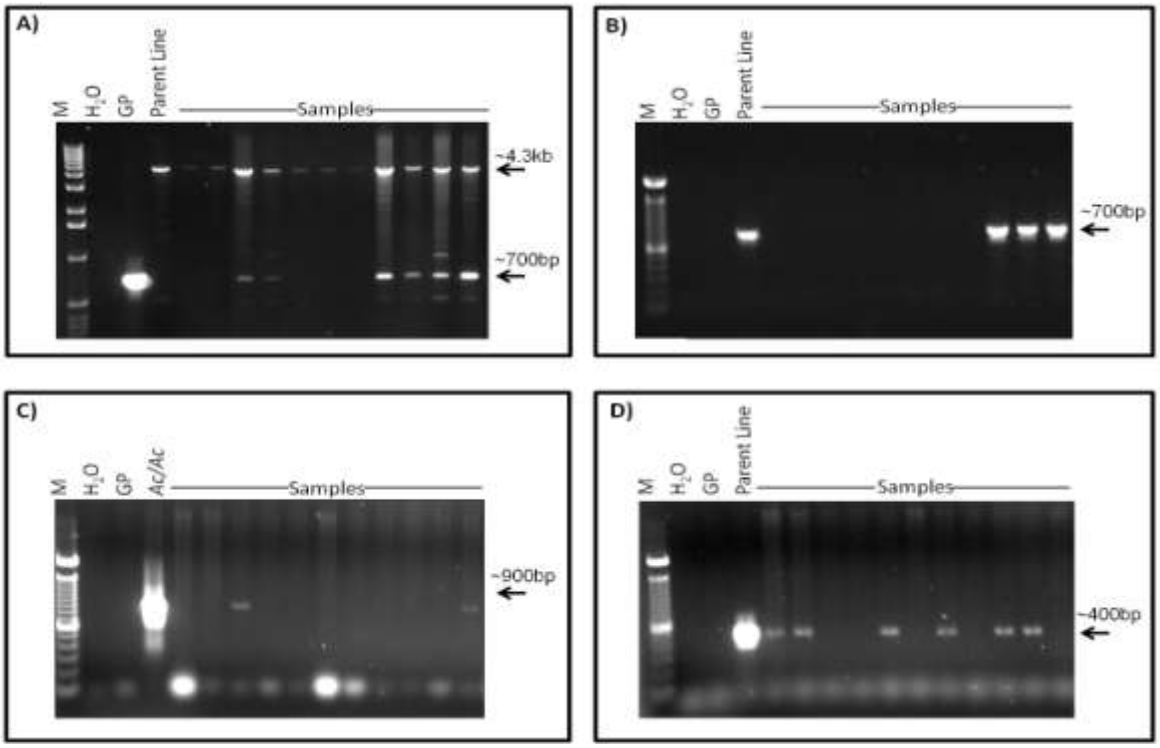


Figure 4.7. PCR analyses of regenerated plantlets. “M” indicates the DNA ladder on all gels. Water was used as the negative controls for all analyses. Golden promise (GP), the non- transgenic plant, was used as the negative controls for PCR analyses in B), C) and D), but was used as a positive control in A). The “parent line” is the DNA from plants originally used for the *Agrobacterium* transformation, it served as a negative control in A) but as positive controls in B) and D). “*Ac*/*Ac*” is the plant that contains the *Ac*TPase gene. It was used as a positive control in C). Arrows indicate the expected band size of each PCR amplification. A) Empty donor PCR which indicates the transposition of *Ds* away from the original site of insertion. B) Flanking sequence PCR indicates the presence of *Ds* in the original site of insertion. C) *Ac* PCR indicates the presence of *Ac*TPase in the genome. D) *Ds* PCR indicates the presence of *Ds* transposon in the genome.

4.4.6. GFP expression and *Ds* transposition

As indicated in Table 4.3, *Ds* transpositions were observed in both GFP and non-GFP expressing embryos. The frequencies of *Ds* transpositions in GFP expressing embryos are 10% (4/40), 37.1% (13/35) and 29.3% (17/58), whereas in non-GFP expressing embryos these frequencies are 11.8% (2/17), 9.1% (1/11) and 50% (8/16) for TNP -13, -29 and -79, respectively. Although low in number, the incidence of transpositions in non-GFP expressing tissues may be due to the inability to detect low level of GFP expression at the early stage. These tissues were considered as non-GFP expressing. The presence of *GFP*/*Ac*TPase in these tissues can be confirmed by PCR analysis. Our preliminary PCR analysis indicates the presence of *Ac*TPase in some plants originating from non-GFP expressing tissues (Table 4.3). Detection of GFP expression is relatively difficult due to the size of the callus and also the low *Ac*TPase expression. However, decreased expression of *Ac*TPase has been reported to elevate *Ds* transpositions in barley and maize, also known as the dosage effect (McElroy et al., 1997; Fedoroff, 1989). Indeed, we observed that the total *Ac*TPase integration frequencies are 17.5% (10/57), 10.8% (5/46) and 4.7% (3/64) for TNP-13. -29 and -79, respectively. Hence, the increased number of *Ds* transpositions in the TNP-79 line in combination with low *Ac*TPase integration supports the argument of the dosage effect.

Nonetheless, preliminary results have indicated the success of reactivating *Ds* transposons using extra-chromosomal expression of *Ac*TPase. The total percentage of *Ds* transpositions occurring after the transient expression of *Ac*TPase is 10.5% (6/57), 30.4% (14/46) and 33.8% (25/64) for TNP-13, -29 and -79, respectively. This frequency was calculated based on the total number of new *Ds* transpositions regardless of GFP expression against the total number of regenerated plantlets for each line. An increased occurrence of *Ds* transpositions were observed compared with *Ds* transpositions generated through cross breeding methods. These increased frequencies of transpositions will greatly facilitate the generation of new *Ds* insertion lines.

The combination of these results clearly indicated that *Ac*TPase is expressed extra-chromosomally during *Agrobacterium* transformation. In addition, transient expression of *Ac*TPase can facilitate and increase the number of *Ds* transpositions.

More importantly, this experiment assessed the possibility of reactivating *Ds* transposons with defective terminal inverted repeats through transient expression of *Ac*TPase. According to the calculated frequencies of *Ds* reactivation, 10.5% of *Ds* transpositions containing defective terminal inverted repeats (TIRs) were observed in the TNP -13. However, using conventional methods no transpositions were observed for this line in previous studies (Singh et al., 2006). According to Singh et al. (2006), *Ds* transposons with damaged TIRs cannot be reactivated.

Based on the preliminary results obtained from this study, the analyses of plantlets regenerated from TNP-13 indicated that *Ds* transposon was reactivated although the TIRs have been damaged. However, studies using conventional methods indicated the inability of *Ds* with damaged TIRs to transpose or a general reduction of 3800 times in *Ds* transposon with TIRs containing a hampered nucleotide (Xiao and Peterson, 2002). In this study, TNP-13 was observed to be able to transpose. This may be explained by the change in methylation patterns during tissue culture. Reactivation of methylated *Ds* transposons has been observed during tissue culture due to demethylation of the TIRs (Kim et al., 2002; Izawa and Shimamoto, 1999). The whole genome methylation pattern has been reported to be reshuffled during tissue culture of plants (Bednarek et al., 2007). Reprogramming of the methylation pattern during tissue culture may allow the *Ac*TPase to access the *Ds* transposon (Brettell and Dennis, 1991) and facilitate, *Ds* transpositions containing damaged TIRs.

Table 4.3. Compilation of PCR data in regards to GFP expression. Total GFP expressing plants, total *Ds* transpositions and total *Ac*TPase integration of each plant lines TNP -13, -29 and -79 are indicated in the table with regards to the expression of GFP.

	Total GFP expressing plants		**Total *Ds* transpositions**		**Total *Ac*TPase integration**	
	GFP (+)ve	GFP (-)ve	GFP (+)ve	GFP (-)ve	GFP (+)ve	GFP (-)ve
TNP -13	40	17	4	2	8	2
TNP -29	35	11	13	1	5	0
TNP -79	58	16	17	8	2	1
Total	133	44	34	11	15	3

4.5. Conclusions

Based on preliminary results, transient extra-chromosomal expression of *Ac*TPase can be used to reactivate *Ds* transposons. The main objective was to develop a new method for *Ds* reactivation by testing the viability of extra-chromosomal expression of *Ac*TPase which was achieved through this current study. The reactivation frequency of approximately 30% was observed in *Ds* transposon (*DsT*) lines with perfect TIRs and 10% in *DsT* lines with imperfect TIRs. These frequencies are greatly elevated compared to *Ds* transpositions generated through conventional methods (11-17% for *DsT* with perfect TIRs and ~0% for *DsT* with imperfect TIRs) (Singh et al., 2006; Xiao and Peterson, 2002). In addition, a negative correlation between the frequency of *Ds* transposition with the frequency of *Ac*TPase integration was observed. This observation elucidates that minimal expression of *Ac*TPase is sufficient for *Ds* transposition, and supports previous reports on the dosage effect of *Ac*TPase expression with *Ds* transpositions.

Since this is one of the few reports regarding extra-chormosomal expression of *Ac*TPase to reactivate the *Ds* transposons, preliminary results have indicated the possibility of using this method for *Ds* reactivation. However, further modifications for this method are required. Some suggestions include screening for GFP fluorescence on the 1^{st}, 2^{nd}, and 3^{rd} days post inoculation (dpi) to verify immature embryos that exhibit transient GFP expression. This is based on literature confirming the maximum transient expression of GFP fluorescence to be from 1.5 to 3 dpi (Dhillon et al., 2009). Also, to further improve the method of GFP detection, RFP can be used. This is because auto-fluorescence emitting from damaged plant tissues interferes with GFP screening, secondary metabolites produced in damaged tissues emits wavelengths that are similar to GFP. Therefore, the red fluorescent protein (RFP), which has a different emission wavelength can be substituted for the use of GFP in plant tissue screening.

The successful utilization of this method will facilitate the generation of new lines by reactivating TNP-29 and -79 *Ds* transposons. Conventional breeding method for *Ds* reactivation requires laborious crossing and generation of F_2

plants. *In vitro* activation of the *Ds* transposon will significantly reduce the amount of effort and time to generate and screen for new *Ds* transpositions. In addition to the increased *Ds* transposition frequency, new *Ds* insertion lines can be directly obtained from the regenerated plantlets. The process can be done in 3 months instead of 2 generations using the conventional methods. This effort would expedite the process of creating new *Ds* transposition, such as the development of lines to saturate the malting quality QTLs on chromosome 4H in barley.

Connecting Statement

Significant numbers of new *Ds* insertion lines were generated through studies conducted in previous chapters (Chapters 3 and 4). Since, functional analyses of these lines require information regarding the genomic regions tagged by *Ds* transposons, flanking sequences need to be obtained. However, current methods to obtain *Ds* flanking sequence information are very inefficient and expensive. This is exacerbated due to the size and complexity of the barley genome.

As described in Chapter 3, nested inverse PCR (iPCR) has been used for the generation of flanking sequences in crop plants. However, due to inconsistencies, extended time and cost required for this procedure, a new method is required for efficient application in large genome cereals. A new PCR-based method, high efficiency thermal asymmetric interlaced PCR (HE- TAIL PCR), was designed based on existing TAIL PCR protocols. The total genomic DNA, experiment time and costs required for generating flanking sequences by HE-TAIL PCR are greatly reduced as compared to iPCR and normal TAIL-PCR methods. Using this new approach, we have successfully generated reliable fragments of flanking sequences adjacent to *Ds* insertions.

The results of this study were compiled in a manuscript which was recently published in the Journal of Plant Molecular Biology and Biotechnology. I have performed all the work described in this chapter under the guidance of Dr. Jaswinder Singh, including the design of primers, the modifications of PCR programs and the evaluation and analyses of HE-TAIL PCR. Funding for this project was provided by Dr. Jaswinder Singh through BMBRI and NSERC-CRD.

Chapter 5

High-Efficiency Thermal Asymmetric InterLaced (HE-TAIL) PCR for amplification of *Ds* transposon insertion sites in barley

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5.1. Abstract

Thermal Asymmetric Interlaced PCR (TAIL PCR) has been used in many different species to isolate flanking sequences adjacent to known sequences. This method has always been a challenge in large genome species, therefore alternative methods have been employed to obtain the flanking sequences. However, these methods are expensive and laborious. Here, we have devised a new and improved method to obtain flanking sequences from the barley genome. The proposed method is named as the High-efficiency Thermal Asymmetric Interlaced PCR (HE-TAIL PCR). We have introduced a new 15-mer sequence from the green fluorescent protein (*GFP*). The new primers efficiently generated transposon flanking sequences in the newly generated barley *Ds* insertion lines as compared to previously reported hiTAIL PCR by Liu and Chen (2007). Using TAIL PCR, minimal manipulation of genomic DNA is required and large number of samples can be performed at the same time, increasing the efficiency of PCR based amplification of flanking sequences. This HE-TAIL PCR method has effectively introduced the many benefits of TAIL PCR into large genome cereals research.

5.2. Introduction

Polymerase Chain Reaction (PCR) has been effectively used as means to understand the biology of many organisms. This robust method had been modified into many different versions; one major modification is used to study adjacent sequences from known transposon or T-DNA sequences. Since standard PCR cannot be used for this purpose, there are 3 major types of PCR based modifications that have been employed to obtain the flanking sequences (Wang et al., 2007). The most commonly used methods include randomly primed PCR (Liu et al., 2007; Mullins et al., 2000), inverse PCR (Liang et al., 2008; Singh et al., 2006), and ligation mediated PCR (YuanXin et al., 2003; Schmidt et al., 2001). Complete review on the different types of PCR based methods to generate flanking sequence amplification has been recently reviewed by Tonooka

and Fujishima (2009). The Thermal Asymmetric Interlaced PCR, also known as the TAIL PCR, falls into the randomly primed PCR category (Wang et al., 2007) and is the only method that is entirely based on PCR (Liu et al., 2007). This method has always been favored in many laboratories to obtain flanking sequences due to its robustness and ease to perform (Zhou et al., 2010; Kong et al., 2009; Pillai et al., 2007). TAIL PCR allows for the easy manipulation of large number of DNA samples and the potential to perform automation to obtain flanking sequences (Singer and Burke, 2003). Modifications to the TAIL PCR can be done accordingly as per specific needs of individual laboratories. One such application is TAIL PCR based strategies to amplify complex GC rich regions (Zhou et al., 2010). TAIL PCR has been extensively used in gene tagging and functional genomics studies. This strategy has been successfully used to isolate important genes in *Arabidopsis* (Kuromori et al., 2004) and rice (Kolensik et al., 2004) where large collections of transposon and T-DNA insertions have been generated.

Recently, similar transposon based functional genomics resources have been developed in barley for gene cloning (Singh et al., 2006), which requires an efficient strategy for isolation of flanking sequences from *Dissocation* (*Ds*) insertions. Similar to other species such as *Arabidopsis* and rice, Inverse PCR (iPCR) and TAIL PCR strategies have been employed to isolate flanking sequences adjacent to *Ds* insertions in barley (Cooper et al., 2004). However, these methods are inefficient to perform high throughput generation of sequences in large genome plants species like barley and wheat (Cooper et al., 2004). Compared to large genome cereals, *Arabidopsis* and rice both have a small genome size of approximately 157 Mbps (Bennett et al., 2003) and 430 Mbps (Sasaki and Burr, 2003) respectively. Barley has a genome size of 5000 Mbps, which is approximately 32 times larger than the model plant, *Arabidopsis,* and approximately 12 times larger than rice. It has been reported that genome size negatively affects the PCR amplification potential (Garner, 2002). As a result, laboratories working on large genome cereals such as barley and wheat mostly resort to other methods such as the iPCR to obtain flanking sequences (Liu et al.,

2009; Singh et al., 2006). However, iPCR is a complicated, laborious and expensive method which involves digestion and ligation steps prior to PCR amplification cycles.

The High Efficiency Thermal Asymmetric Interlaced PCR (HE-TAIL PCR) method that we propose is an improved version of the hiTAIL PCR published by Chen and Liu (2007). We introduced a unique 15-mer sequence to the degenerative primers (Liu et al., 2007). Due to the unavailability of complete barley genome sequence, we decided to include a sequence from a heterologous species. We have designed the 15-mer sequence corresponding to the green fluorescent protein (GFP) to avoid non-specific amplification. In all instances, to keep the high specificity amplification of the flanking sequences, we ensured that all specific primers and the 15-mer primer have a high melting temperature (Tm 62˚C-68°C). This modification has allowed us to obtain the maximum number of flanking sequences from the barley *Ds* transposon insertions, thereby creating a new and efficient method to obtain flanking sequences in large genome cereals.

5.3. Materials and Methods

The general overview of experimental method is shown in Figure 3.1.

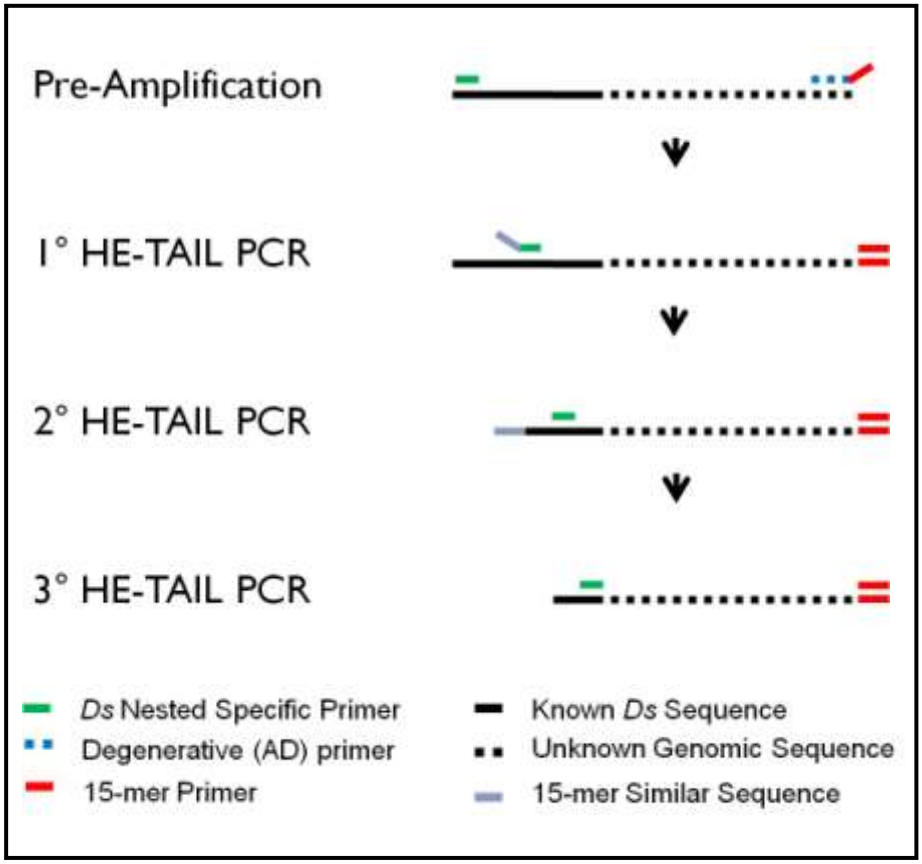

Figure 5.1. Strategy for HE-TAIL PCR.

5.3.1 Barley transposon insertion lines

A population of *Ds* insertion lines has been generated at McGill University, Ste-Anne-de-Bellevue, Canada. The strategies used to develop barley insertional mutagenesis resource have been discussed in Chapters 3 and 4. From the repository of *Ds* containing lines, seven *Ds* barley lines including two known and 5 unknown *Ds* insertion sites were selected. Each new single copy *Ds* insertion line (TNP lines), was analyzed by basic PCR analysis and Southern blotting as described previously in chapter 3 before proceeding to HE-TAIL PCR.

5.3.2. HE-TAIL PCR

Takara ExTaq DNA polymerase, the supplied 10X PCR buffer containing 10 mM $MgCl_2$, and 2.5 mM dNTPs (Takara-Bio, Dalian, China) was used for HE-TAIL PCR. Genomic DNA of the selected barley TNP lines were subjected to 4 separate runs of HE-TAIL PCR. These runs include pre-amplification, primary amplification, secondary amplification and tertiary amplification respectively. The first 3 PCR runs were modified from Liu and Chen (2007) and Singer and Burke (2003). The PCR were performed using GeneAmp® PCR System 9700 by Applied Biosystems.

5.3.2.1 Pre-Amplification

The pre-amplification reaction mix contained 1X ExTaq DNA polymerase buffer, 0.5U of ExTaq DNA polymerase, 0.2 mM dNTPs and, 1.0 µM degenerative primers (QTLAD1-1, 1-2, 1-3 or 1-4), 0.3 µM of the first specific nested primer (JNosF), and 20-30 ng of genomic DNA, adjusted to a total volume of 20 µl per reaction (Liu and Chen 2007). The PCR program included an initial denaturation at 93°C for 2 min and 95 °C for 1 min. This was followed by 11 cycles of 94°C for 30 s, 60°C for 60 s and 72°C for 3 min. The reaction was then heated to 94°C for 30 s before cooling to 25°C for 2 min. Then, a gradual ramping up to 72°C at a rate of 0.5°C/s was performed. The reaction remained at 72°C for 3 min followed by 26 cycles of standard PCR program at 94°C for 20 s, 58°C for 60 s and 72°C for 3 min followed by final elongation at 72°C for 5 min.

5.3.2.2. Primary Amplification

The Pre-Amplification HE-TAIL PCR product was diluted 40 fold before subjecting to the primary amplification. The reaction mix contained 1X ExTaq DNA polymerase buffer, 0.6U of ExTaq DNA polymerase, 0.2 mM dNTPs and, 0.3 µM of the 15-mer primer (QTAC-1), 0.3 µM of the second specific nested primer (QTPF2) and 1 µl of 40 times diluted pre-amplification reaction, adjusted to a total volume of 25 µl per reaction. The PCR program for the primary TAIL PCR included 2 cycles of 94°C for 20 s, 65°C for 60 s, 72°C for 3 min, followed by

14 cycles of 94°C for 20 s, 68°C for 60 s, 72°C for 3 min, 94°C for 20 s, 50°C for 60 s and 72°C for 3 min and a final elongation was done at 72°C for 5 min.

5.3.2.3. Secondary Amplification

After the Primary TAIL PCR, the primary amplification product was diluted 5 times for the secondary amplification. The secondary amplification of the HE-TAIL PCR cocktail contains 1X ExTaq DNA polymerase buffer, 0.6U of ExTaq DNA polymerase, 0.2 mM dNTPs and, 0.3 µM of the 15-mer primer (QTAC-1), 0.3 µM of the third specific nested primer (JDsB-3) and 1 µl of 5 times diluted primary amplification product, adjusted to a total volume of 25 µl per reaction. The secondary TAIL PCR program include 14 cycles of 94°C for 20 s, 68°C for 60 s, 72°C for 3 min, 94°C for 20 s, 50°C for 60 s, and 72°C for 3 min, followed by a final elongation at 72°C for 5 min.

5.3.2.4 Tertiary Amplification

This cycle was used only if the secondary round did not yield sufficient amplification. Products from the secondary amplification were diluted 25 to 1000 fold depending on the intensity of the tertiary amplification. The reaction mix contained 1X ExTaq DNA polymerase buffer, 0.6U of ExTaq DNA polymerase, 0.2 mM dNTPs and, 0.3 µM of the 15-mer primer (QTAC-1), 0.3 µM of the fourth specific nested primer (JDsB-3), and 1 µl of diluted secondary amplification reaction, adjusted to a total volume of 50 µl per reaction. The tertiary TAIL PCR program include 94^{o}C for 20 s, followed by 36 cycles of 94°C for 20 s, 56°C for 60 s and 72°C for 3 min, followed by a final elongation at 72°C for 5 min.

5.3.3 Gel electrophoresis and sequencing

All HE-TAIL PCR products were visualized on a 0.8% agarose gel. PCR products were directly sequenced without performing any purification at the McGill University and Génome Québec Innovation Center.

Table 5.1. HE-TAIL PCR Program. He-TAIL PCR program used on an Applied Biosciences Gene Amp® PCR system 9700, modified from Liu and Chen, (2007) and Singer and Burke, (2003). (a) Optional Tertiary He-TAIL PCR amplification.

Pre-amplification			**Primary TAIL-PCR**			**Secondary TAIL-PCR**			**Tertiary TAIL-PCR(a)**		
Step	Temperature (°C)	Time	Step	Temperature (°C)	Time	Step	Temperature (°C)	Time	Step	Temperature (°C)	**Time**
1	93	2:00	1	94	0:20	1	94	0:20	1	94	0:20
2	95	1:00	2	65	1:00	2	68	1:00	2	94	0:20
3	94	0:30		72	3:00	3	72	3:00	3	56	1:00
4	60	1:00	4	Go to Step 1	1 time	4	94	0:20	4	72	3:00
5	72	3:00	5	94	0:20	5	50	1:00	5	Go to Step 2	35 times
6	Go to Step 3	10 times	6	68	1:00	6	72	3:00	6	72	5:00
7	94	0:30	7	72	3:00	7	Go to Step 1	13 times	7	4	∞
8	25	2:00	8	94	0:20	8	72	5:00			
9	Ramping to 72	0.5C/s	9	50	1:00	9	4	∞			
10	72	3:00	10	72	3:00						
11	94	0:20	11	Go to Step 5	13 Times						
12	58	1:00	12	72	5:00						
13	72	3:00	13	4	∞						
14	Go to Step 11	25 times									
15	72	5:00									
16	4	∞									

Table 5.2 Primers used for HE-TAIL PCR. The primers were modified from Liu and Chen, (2007). Sequences in bold are sequences specific to the 15mer primer.

Primers	Sequences
QTLAD1-1	5'- **TAGCGGCTGAAGCAC**CTGCAGGCVNVNNNGGAA -3'
QTLAD1-2	5'- **TAGCGGCTGAAGCAC**CTGCAGGCBNBNNNGGTT -3'
QTLAD1-3	5'- **TAGCGGCTGAAGCAC**CTGCAGGCVVNVNNNCCAA -3'
TLAD1-4	5'- **TAGCGGCTGAAGCAC**CTGCAGGCBDNBNNNCGGT -3'
QTAC-1	5'- **TAGCGGCTGAAGCAC** -3'
JNosF	5'- GCGCGGTGTCATCTATGTTACTAGATC -3' (313bp from TIR)
QTPF-2	5'- **TAGCGGCTGAAGC**TGCCTGCAGGAAACGGTCGGGAAACTAGCTC -3' (175bp from TIR)
JDsB-3	5'- TGTATATCCCGTTTCCGTTCCGTT -3' (126bp from TIR)
JIPF8	5'- TATACGAAACGGTCGGTACGG -3' (48bp from TIR)
QDs1-5	5'- CCCGTCCGATTTCGACTTTAACCC -3' (181bp from TIR)
QTPR1	5'- **TAGCGGCTGAAGC**TGCCTGCAGGACCGGATCGTATCGGTTTTCG -3' (158bp from TIR)
JIPR5	5'- TTCGTTTCCGTCCCGCAAGT -3' (89bp from TIR)
QDs4-5	5'- CGACCGTTACCGACCGTTTT -3' (34bp from TIR)

5.4. Results and discussion

5.4.1. HE-TAIL PCR primers and program design

Many modifications of TAIL PCR and its variations have been done over the years to isolate genes from numerous organisms (Zhou et al., 2010; Kong et al., 2009; Wang et al., 2007). Although TAIL PCR is a highly versatile method for generating flanking sequences, this method is inefficient for complex large genome cereals. To further improve TAIL PCR for large genome cereals such as barley, we have modified the methods of Liu and Chen (2007) and Singer and Burke (2003), and named it as the High efficiency Thermal Asymmetric Interlaced PCR (HE-TAIL PCR). In our protocol, the major modification is in the primer design along with modifications in the PCR program.

Barley has a complex genome with a size of approximately 5000 Mbps. As with most large genome cereals, such as wheat and oats, the genome of this species has not been completely sequenced. Hence, good primer design for HE-TAIL PCR is essential for the successful generation of flanking sequences in this crop plant. The 16-mer primer proposed by Liu and Chen (2007) was optimized to be effectively used in rice. Unfortunately, bioinformatic analysis using the NCBI database indicates that this primer has high similarity with sequences from a large number of organisms ranging from prokaryotes to eukaryotes. Since the complete sequence of large genome cereals is not available, we speculated that this sequence could have similarity with some sequences in the genome of crop plants such as barley. Here, we propose to use a 15-mer sequence which has been designed from a heterologous sequence of the green fluorescent protein (GFP). A BLAST analysis for short input sequences was performed to verify the absence of 3' end homology of the 15-mer primer with the available sequence of barley and wheat. With this modification we can effectively eliminate most non-specific binding of the 15-mer primer, hence increasing the specificity of the primer in HE-TAIL PCR in barley. Except for the modification described above, the degenerative primers used were the same as described by Liu and Chen (2007). All the specific primers designed from the *Ds* construct have a melting

temperature above 68°C using the Tm calculation of Tm = 69.3 + 41 X GC% - 650/L where L = primer length.

The comparative amplification efficiency of PCR using our newly designed and previously reported primers by Liu and Chen (2007) is shown in Figure 5.2. Lanes on the left (1-8) represent HE-TAIL PCR products using primers from Liu and Chen (2007). Lanes on the right (9-16) represent HE-TAIL PCR products using primers designed from GFP. As shown in Figure 5.2 - C, enhanced amplification of HE-TAIL PCR was obvious. PCR products obtained from lanes 9 through 16 compared to lanes 1 through 8 were significantly improved. This result clearly support our hypothesis that the primers designed using the *GFP* sequence is certainly superior than the previously reported primers (Liu and Chen, 2007) particularly for barley, a large genome cereal. Consistent with the previous report from Liu and Chen (2007), we have successfully obtained fragments that are relatively larger (>300bp) than that obtained with normal TAIL PCR products (Liu et al., 1995). Sequences obtained from HE-TAIL PCR amplicons were in the range of 800 bp to 1000 bp. BLAST analyses showed that the *Ds* insertions were in the genic region as shown previously (Singh et al., 2006). *Ds* insertion site structure for each sequence is shown in Figure 5.3. The genes tagged by *Ds* in the current study include ABC transporter (Sample A; E value 1e-16 and sample C; E value: 4e-93), glutamine synthetase gene (sample B; E value 3e-25) and BAC 519K7 hardness locus region (sample D; E value 5e-61).

Besides performing the comparison of primers for HE-TAIL PCR to obtain the 3'end of the flanking sequence, HE-TAIL PCR was also successfully performed for the 5' side of *Ds* insertions using newly generated primers (Figure 5.2 - D). Generation of flanking sequences using this modified TAIL PCR is now routinely being used in our laboratory for gene discovery in barley.

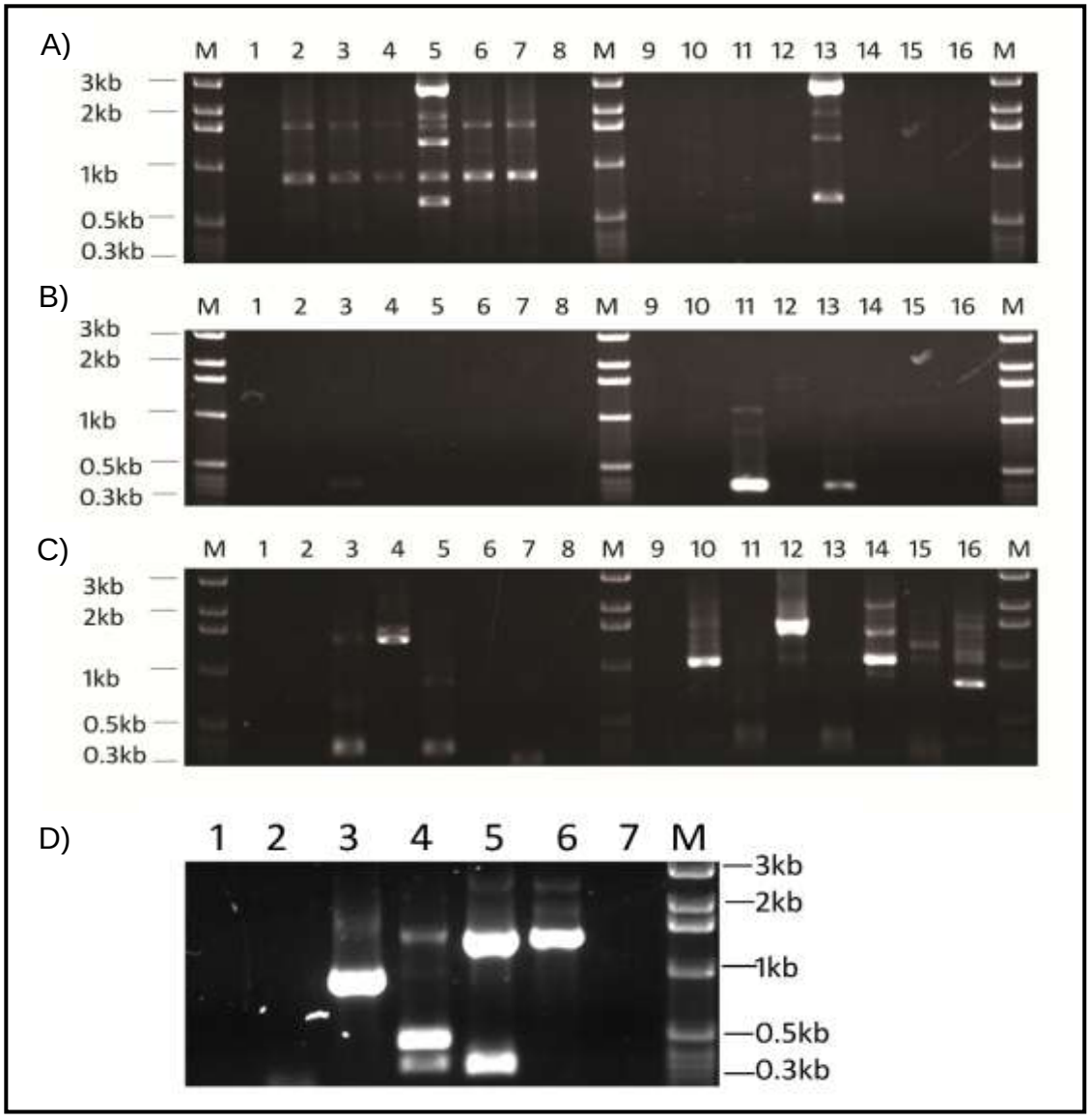

Figure 5.2. Products of primers for the HE-TAIL PCR. PCR amplification using primers by Liu and Chen, (2007) (lanes 1-8) and using new primers designed for this study (lanes 9-16). (A) Pre-amplification run of HE-TAIL PCR. (B) Primary HE-TAIL PCR run. (C) Secondary HE-TAIL PCR run. "M" indicates the DNA size marker. Lanes 1 to 8 and 9 to 16 are the samples in same order. Lane 1: negative control (water), Lane 2: positive control (TNP 79- known *Ds* insertion), Lane 3: 2nd positive control (TNP29- known *Ds* insertion), Lane 4-8: individuals with unknown *Ds* insertions. (D) Secondary HE-TAIL PCR run for the 5' end of *Dissociation* (*Ds*), lanes 1-2: negative controls, Lanes 3-7: individuals with unknown *Ds* insertions

Figure 5.3. *Ds* insertion site structure. (A) and (B) are examples of 5' side flanking sequences obtained from the 5' HE-TAIL PCR. (C) and (D) are examples of 3' side flanking sequences obtained from the 3'end HE-TAIL PCR. Bold letters represent the *Ds* construct; boxed letters represent the terminal inverted repeats (TIRs) and underlined letters represent 8bp duplicates at the *Ds* insertion site. The first 100bp of flanking sequences were shown. The TIRs of (C) are found to be damaged.

5.4.2. Other factors

As with all TAIL PCR products, the end product might generate many bands with different sizes. This is due to the degenerative primer that randomly primes in the genome. Direct sequencing may be employed in this instance because all fragments generated are essentially from the same sequence. Hence, further purification is not needed, decreasing the amount of workload and reagents to be used for preparation of the sample.

As mentioned above, numerous methods have been employed to determine flanking sequences. HE-TAIL PCR offers several distinct advantages over iPCR, currently used method to obtain flanking sequences from barley genome. HE-TAIL PCR can be fully automated as no further manipulation of DNA is required. Only a minute amount of genomic DNA, 20-30 ng, can be used immediately for HE-TAIL PCR. Whereas for iPCR analysis, the amount of genomic DNA required is at least 25 times more due to additional DNA manipulation in this protocol.

As for large scale generation of flanking sequences, the cost and time of the method chosen for generation of these sequences should also be taken into consideration. We observed that the cost of performing HE-TAIL PCR is approximately 10 times lower than iPCR. As per our calculations, the cost of HE-TAIL PCR was $2.60 per sample as compared to $19.60 for iPCR. We also observed that direct sequencing of HE-TAIL PCR products significantly made this system efficient and compatible for full automation from DNA extraction to sequencing. However, in the case of iPCR it is challenging to do so and alternative restriction enzymes are generally required to obtain additional and better flanking PCR fragments.

Stating the above facts regarding iPCR, it is important to note that HE-TAIL PCR is not fool-proof. In HE-TAIL PCR, it is possible that one degenerative primer might not be able to produce flanking sequence fragments for all samples. This imperfection can be improved by using 2 or more degenerative primers at the same time thus increasing the chances of obtaining a flanking sequence. TAIL PCR also has been shown to have an efficiency of up to 93.3% success rate (Liu et al., 2007). However, primers must be standardized as they show variation from one species to the other.

5.5. Conclusions

Our main objective was to develop a new and efficient method of generating flanking sequences had been successfully achieved. The design of a new 15 mer sequence using a heterologous sequence from GFP has significantly improved the amplification of HE-TAIL PCR fragments. We introduced the use of HE-TAIL PCR as a better option of TAIL PCR to generate flanking sequences in large genome cereals such as barley and wheat. This method will greatly improve the efficiency and cost of generating flanking sequences in large genome cereals where manipulation of DNA is relatively difficult.

Chapter 6

General Discussion, Conclusions, Future Studies and Contributions

6.1 General discussion

6.1.1 Generation of new *Ds* insertion lines

Transposon mutagenesis is very valuable for gene cloning and functional genomics studies. Creation of *Ds* insertion mutants has a great potential for gene tagging and saturation mutagenesis in barley. Characterization of mutant alleles provides valuable insights into the genetics of normal physiological functions (Singh et al., 2006). In this study, a total of 47 new transposition events were generated from candidate *Ds* lines associated with malting quality QTLs. The reactivation frequency of *Ds* was determined to be consistent in the expected range of 10-11%. These results concur with previous publications regarding the *Ds* reinsertion frequencies which range from 11% to 17% in barley (Singh et al., 2006; Koprek et al., 2000).

The identification of 6 new *Ds* flanking sequences confirmed the preference of *Ds* insertions into genic regions. Although the relationship between these genes with malting quality still needs to be investigated, basic bioinformatics analysis identified malting quality related genes, including a *Ds* knockout in the *β-amylase* gene involved in sugar degradation process. Sugar degradation is one of the important malting quality parameters during the malting process (Swanston et al., 2002), therefore the new *Ds* knockout in the *β-amylase* gene will facilitate to understand this process thoroughly. Since basic bioinformatic analysis only suggests the putative gene functions, subsequent analysis such as reverse genetics remains to be done to further confirm the gene functions.

These findings suggest the usefulness of *Ds* reactivation from TNP-29 and -79 to target malting quality traits and have also confirmed the ability of the

Ac/Ds transposon system to be effectively utilized as a gene search engine in barley for functional genomics studies.

6.1.2 Improved method for generation of new *Ds* insertion lines

Although the reactivation of *Ds* transposon by conventional methods can be utilized for transposon tagging purposes, partial or complete silencing of *Ac*TPase expression in advanced generations was observed (Koprek et al., 2000). The lack of reliable *Ac*TPase expressing lines prompted our intentions to develop *in vitro* methods where *Ac*TPase can be provided extra-chromosomally. To serve this purpose, a new binary construct, pCambia-pAct*GFP*-pUbi*Ac* was developed in the present study.

Utilizing this construct, expression of *Ac*TPase has been associated with expression of GFP. Consistent with previous reports, maximum transient expression of green fluorescent protein (GFP) was visualized on the 3rd day post inoculation (dpi) during the extra-chromosomal expression of transgenes using *Agrobacterium* (Upadhyaya et al., 2006) and biolistic methods (Dhillon et al., 2009). In this study, GFP expression was used as an indicator for *Ac*TPase activity and appropriate tissues were identified after the 3rd, 10th and 17th dpi. Based on our observations, GFP fluorescence after the 3rd dpi clearly indicated new *Ds* transpositions. Hence, GFP expressing from 1.5 to 3 dpi could be the major time points to determine the transposition of *Ds*. A recent study showed that there is an accumulation of transgene expression from 1.5 to 3 dpi (Dhillon et al., 2009). Therefore, screening of transient GFP expression should be done on the 1st, 2nd and 3rd days post inoculation.

Preliminary data clearly indicate the success in reactivating of *Ds* transposons (*DsT*) *in vitro*. The reactivation frequency of 30% was observed for *Ds* insertions with perfect terminal inverted repeats (TIRs) and 10% *DsT* with imperfect TIRs. These reactivation frequencies are much higher than previously reported frequencies obtained through cross breeding methods (11-17% for *DsT* with perfect TIRs and ~0% for *DsT* with imperfect TIRs) (Singh et al., 2006; Xiao and Peterson, 2002). Reactivation of *Ds* elements from a line containing

damaged TIRs is an important observation of this study. Although precise reasons for this activity are not known, however, we speculate that it may be associated with the resetting of the TIRs methylation status during tissue culture.

Besides, some *Ds* transpositions were observed in plants regenerated from non-GFP expressing embryos. This may be due to undetectable or low GFP expression in small tissues, signifying the low expression of *Ac*TPase. However, low expression of *Ac*TPase might be sufficient for activation of the *Ds* element. This observation is in agreement with previous reports, where decreased expression of *Ac*TPase was observed to elevate *Ds* transpositions in barley and maize (McElroy et al., 1997; Fedoroff, 1989). In addition, we observed an inverse correlation between the total *Ac*TPase integration frequencies with *Ds* transposition frequencies.

The combination of these results clearly indicated that *Ac*TPase can be expressed extra-chromosomally through *Agrobacterium* transformation. Also, transient expression of *Ac*TPase can facilitate and increase the number of *Ds* transpositions. Utilization of the method will create new *Ds* insertion lines efficiently. Moreover, *Ds* insertions with damaged TIRs located near important genes can be reactivated using *in vitro* extra-chromosomal expression of *Ac*TPase.

The successful development of this method can greatly facilitate the generation of new *Ds* insertion lines by reducing the time in half, because *Ds* reactivation through the *in vitro* method can be detected following the regeneration process which is only approximately three months. Also, stable *Ds* insertion lines can be efficiently generated in a short period of time without the need to segregate the *Ac*TPase gene. Taken together, a large number of *Ds* knockouts can be generated for functional genomics resources in barley.

6.1.3 Improved method for generation of flanking sequences

Further, to maximize the utility of newly developed transposon mutants, structure of *Ds* insertion sites in the genome need to be determined. This is directly related with the efficiency of generating *Ds* flanking sequences.

Unfortunately, current methods such as the nested inverse PCR (iPCR), used in large genome cereals, is inefficient, expensive and laborious. In this study, a new method, the high efficiency thermal asymmetric interlaced PCR (HE-TAIL PCR), was devised to expedite the functional genomics studies in barley.

The introduction of a unique 15-mer in our TAIL-PCR experiments has significantly improved the amplification of flanking sequences. The original 16-mer primer proposed by Liu and Chen (2007) was optimized to be effectively used in rice. Unfortunately, bioinformatics analysis using the NCBI database indicates that this primer has high similarity with sequences from a large number of organisms ranging from prokaryotes to eukaryotes. Therefore, the newly designed 15-mer sequence was designed based on a heterologous sequence from the *GFP.* Absence of the 3' end homology of the 15-mer sequence with the *GFP* sequence was designed with available sequences of barley and wheat. This design has considerably improved the performance of normal TAIL PCR. We have successfully obtained larger fragments (>300bp) than normal TAIL PCR products (Liu et al., 1995) and have greatly facilitated in the generation of reliable flanking sequences for the determination of *Ds* insertion sites in the barley genome.

This method incorporates the benefits of PCR, such as the utilization of small amounts of DNA, increased efficiency and reproducibility. Only a minute amount of genomic DNA, 20-30 ng, can be directly used for HE-TAIL PCR. Whereas for iPCR analysis, the amount of genomic DNA required is at least 25 times more due to additional DNA manipulation. We also observed that the cost of performing HE-TAIL PCR is approximately 10 times lower than iPCR.

In addition, HE-TAIL PCR can be fully automated as no further manipulation of DNA is required. Also, flanking sequence can be obtained without further purification, decreasing the amount of workload and reagents to be used for preparation of the sample.

In short, the successful utilization of HE-TAIL PCR has effectively reduced the time and cost to generate flanking sequences. Reproducible results showed the reliability and consistency of this method. Therefore, routine use of

this method will have broader implications for large genome cereals for which the amplification of flanking sequences is difficult. Successful utilization of this method will contribute to the efficient generation of more new *Ds* insertion lines. A manuscript containing complete aspects of this new strategy was published in the Journal of Plant Molecular Biology and Biotechnology (Tan and Singh, 2011)

6.2. Conclusions

The maize *Ac/Ds* transposon system has been used in barley as a gene tagging tool. To identify new *Ds* insertions, we reactivated *Ds* transposons (*DsT*) in single copy *Ds* insertion (TNP) lines, TNP-29 and -79, to saturate two important malting quality QTLs on chromosome 4H. Reactivation of *Ds* transposon from these lines was achieved using two different approaches. The main approach was carried out by crossing these TNP lines with a transposase (*Ac*TPase) expressing line (25-B) (Chapter 3). As conventional approach is laborious and time consuming, in addition to the use of limited stable *Ac*TPase expressing transgenic lines, a new *in vitro* approach utilizing transient expression of *Ac*TPase for *Ds* activation was devised (Chapter 4). To facilitate the high throughput analyses of *Ds* insertion sites in barley, a new PCR based strategy to obtain *Ds* flanking sequences was also developed (Chapter 5). This new method was named as high-efficiency thermal asymmetric PCR (HE-TAIL PCR). The combination of these methods will contribute to expediting the process of generating new *Ds* insertion lines. Collectively, these efforts will advance our understanding of the important malting quality QTLs in barley.

Conclusively, this thesis has successfully demonstrated our hypotheses: New *Ds* insertion lines can be utilized for gene identification within the malting quality QTLs; transient expression of *Ac*TPase can be utilized for the reactivation of *Ds* transposons and the HE-TAIL PCR-based method can be efficiently utilized to isolate of flanking sequences. Finally, the combination of three experiments contributed to the increased efficiency in generating new *Ds* insertion lines for the saturation mutagenesis of the malting quality QTLs in barley.

6.3 Future directions

In order to saturate QTL2 region and to further understand the genes tagged by the new *Ds* insertion lines, following studies are required in the future:

1. *Development of additional Ds lines from TNP-29 and -79 using conventional and new approaches described in this study*. As QTL2 region is quite large, additional *Ds* lines are required to fully enrich the QTL2 with *Ds* transposons. These lines will facilitate the identification of the gene/genes located in these QTLs.

2. *Molecular mapping of new Ds insertion lines for the saturation of the malting quality QTLs*. Utilizing molecular markers in combination with mapping software, the mapped location of these new *Ds* insertions can be determined. Further experimentation utilizing these new *Ds* loci will determine the association of *Ds* loci with malting quality QTLs.

3. *Gene expression studies on gene/genes identified in the malting quality QTLs.* Gene knockouts produced by *Ds* insertion lines are useful resource for gene expression studies. RNA and protein analysis can be conducted to determine gene functions, especially for gene/genes located in the malting quality QTLs.

4. *Micromalting studies of candidate Ds insertion lines.* Following gene expression studies, appropriate *Ds* homozygous lines must be tested in micromalting experiments. The *Ds* knockouts in the genes influencing malting quality can be further investigated for polymorphism in barley germplasm. Successful identification of polymorphic genes affecting malting quality will facilitate the development of functional markers for breeding programs to develop better malting varieties.

6.4 Contributions to science

The summary of the major accomplishments of my thesis are described below:

1. Generated 47 new *Ds* insertions lines.
2. Identified 6 new genes through transposon tagging.
3. Developed a new efficient *Ds* reactivation method.
4. Devised a high-throughput strategy to generate flanking sequences.
5. Generated a construct for GFP and *Ac*TPase expression.

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