A First Successful Effort of Heterologous Transposons Movement in Oat Genome

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"Oats have been linked to Cinderella, an attractive and productive servant, wholesome and dependent, almost thriving on neglect and disinterest, but overshadowed by more assertive if less attractive step-sisters" *Robert W. Welch, The Oat Crop Production and Utilization (1995)*

Abstract

Common oat (*Avena sativa* L.) is one of the major cultivated cereals and an important nutritional component of human food and animal feed. The recent and rising interest in this crop's healthy components is shaping oat breeding programs' new orientations. However, information regarding oat genetic and genomic resources has lagged, impeding further improvement in oat cultivars. This limitation requires recruitment of functional genomic approaches to explore this genome. In such large genomes, transposon-based functional characterization offers a great potential for gene identification. To develop gene tagging and gene-editing resources for oats, maize (*Zea mays* L.) *Ac/Ds* transposable elements were introduced into the cultivated oat genome. Using a biolistic delivery system, highly regenerative calli derived from mature oat seeds were successfully transformed, both transiently and stably, with several *Ac/Ds* constructs. A *GUS*-gene trap provided initial confirmation of the *Ac/Ds* system' transpositional activity in transformed oat calli. The appearance of blue *GUS* foci indicated the dissociation (transposition) of the *Ds* element from its original location, under *Ac* transposase catalytic activity. Following bombardment, a total of 2035 oat calli pieces were subjected to antibiotic selection. Twenty-four independent, stable transgenic events were obtained, with transformation frequencies of up to 9.5%, and 1.9% for bialaphos and hygromycin selection, respectively. Prior to reactivation of *Ac/Ds* lines, transposase activity in oat *Ac/Ds* transformants was verified by RT-PCR. Since *Ds* is a non-autonomous element, the *Ds* element was reactivated by introducing an *Ac* transposase (*Ac*Tpase) source through either (*i*) generation advancement of oat lines containing both *Ac* and *Ds* elements, or (*ii*) *in vitro* extra-chromosomal activity of *Ac* in the *Ds*-only lines. Transposition frequencies exceeding 15% were achieved through the former (16.9%) and latter (15.9%) techniques. The *Ds* remobilization was followed by histochemical and molecular assays. Sequencing of amplified fragments from the empty donor launch site further confirmed the excision of *Ds* from its original site. The observed amplification of empty donor *Ds* launch sites suggested a high frequency of *Ds* excision (74.6%). Sequencing these footprints showed large deletions and re-structuring of the sequences adjacent to the original *Ds* original, in both plasmids *Ds*Ubi*Bar* and *DsBarGus*. The TaqMan assay for Copy Number Variation (CNV) showed a low copy number of *Ds* insertions in almost all the *Ac/Ds* lines tested. The structure of *Ds* tagged sites was elucidated using inverse PCR, Tail-PCR, and adapter ligation. Twenty-one *Ds* flanking sequences were isolated, for most

of which — apart from the *Ds* insertion found in the GA 20-oxidase 3 (*GA20ox3*) gene — the identify of sequences could not be retrieved.

Our transgenic and transposition lines provide new launching pads for further functional genomic studies in oat. Reactivation of *Ds* element transposition from its original position shows a great potential for gene tagging via *Ds* interruptions/activation as well as editing through footprint insertions and/or deletions generated during transpositions. This will offer the oat breeding community a new tool to develop a better functional understanding of the oat genome.

Résumé

L'avoine commune (*Avena sativa* L.) est une des céréales cultivées majeures, qui constitue une composante nutritive importante pour la consommation humaine et animale. L'intérêt récent et croissant pour cette culture en raison de ses composants sains, a façonné les nouvelles orientations des programmes d'amélioration de sa reproduction. Cependant, en ce qui concerne les ressources génétiques et génomiques, l'avoine a pris du retard, ce qui empêche toute amélioration supplémentaire des cultivars d'avoine. Cette limitation nécessite le recrutement d'approches génomiques fonctionnelles pour explorer ce génome. Dans de tels génomes, la caractérisation fonctionnelle basée sur les transposons offre un grand potentiel pour l'identification des gènes. Afin de développer des ressources de marquage et d'édition de gènes, les éléments transposables *Ac*/*Ds* du maïs (*Zea mays* L.) ont été introduits dans le génome de l'avoine cultivée. En utilisant un système d'administration biolistique, des cals hautement régénératifs dérivés de graines matures ont été transformés avec succès de manière transitoire et stable avec plusieurs constructions *Ac*/*Ds*, Une trappe à gène *GUS* a initialement confirmé l'activité de transposition du système *Ac*/*Ds* dans des cals d'avoine transformés. L'apparition de foyers *GUS* bleus indique la dissociation (transposition) de l'élément *Ds* de son emplacement d'origine, en raison de l'activité catalytique de la transposase *Ac*. Après le bombardement, 2035 cals d'avoine ont été soumis à une sélection d'antibiotique. Vingt-quatre événements transgéniques stables et indépendants ont été obtenus, avec une fréquence de transformation allant jusqu'à 9,5% et 1,9% pour les sélections du bialaphos et de l'hygromycine respectivement. Avant la réactivation des lignes *Ac*/*Ds*, l'activité de transposase *Ac*/*Ds* a été vérifiée par RT-PCR. Puisque *Ds* est un élément non autonome, l'élément *Ds* a été réactivé en introduisant une source de transposase *Ac* (AcTpase), soit (*i*) par le développement de lignées d'avoine générationnelles contenant à la fois des éléments *Ac* et *Ds*, ou (*ii*) par l'activité extra-chromosomique in vitro de *Ac* dans les lignées *Ds* uniquement. Des fréquences de transposition supérieure à 15% ont été obtenue soit, (16,9%) par l'ancienne technique et (15,9%) par la récente technique. En outre, la remobilisation de *Ds* a été suivie des tests histochimiques et moléculaires. Le séquençage des fragments amplifiés du site de lancement du donneur vide a également confirmé l'excision de *Ds*, à partir de son site d'origine. L'amplification observée des sites de lancement de *Ds* de donneurs vides suggèrent une fréquence élevée d'excision de *Ds* (74,6%). Le séquençage de ces empreintes a montré d'importantes délétions et restructurations des séquences adjacentes au site d'origine de *Ds*, dans les deux plasmides *Ds*Ubi*Bar* et *DsBarGus*. Le

test TaqMan pour la variation du nombre de copies (CNV) a montré un faible nombre de copies d'insertion de *Ds* dans presque toutes les lignées *Ac*/*Ds* testées.

La structure des sites marqués de *Ds* a été élucidée à l'aide des méthodes de PCR inversées, Tail-PCR et de ligature d'adaptateur. Vingt et une séquences flanquantes de *Ds* ont été isolées. Dans de nombreuses séquences, l'identité n'a pas pu être récupérée, à l'exception de l'insertion *Ds* trouvée dans le gène GA 20-oxydase 3 (GA20ox3).

Nos lignées transgéniques et de transposition fournissent de nouvelles bases de lancement pour d'autres études génomiques fonctionnelles sur l'avoine. La réactivation de la transposition de l'élément *Ds* à partir de sa position d'origine présente un grand potentiel pour le marquage de gènes via des interruptions/activations de *Ds*, ainsi que pour l'édition via des insertions d'empreinte et/ou des suppressions générées lors des transpositions. Cela offrira à la communauté de reproduction de l'avoine un nouvel outil pour développer une meilleure compréhension fonctionnelle du génome de l'avoine.

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LIST OF ABBREVIATIONS

2,4,5-T	2,4,5-Trichlorophenoxyacetic Acid
2,4-D	2,4-dichlorophenoxyacetic acid
Ac	Activator transposon
Act1	Actin
*Ac*Tpase	Activator Transposase
AFLP	Amplified Fragment Length Polymorphism
Agro	Agrobacterium
ALPCR	Adapter Ligation PCR
APH	Aminoglycoside Phosphotransferase
ARS	(USDA) Agricultural Research Service
BAP	6-Benzylaminopurine
Bar	Bialaphos resistance
BME	β-mercaptoethanol
Cas9	CRISPR-associated protein 9
CIA	Chloroform: Isoamyl Alcohol
cM	centimorgans
CRISPR	Clusters of Regularly Interspaced Short Palindromic Repeats
CTAB	Cetyltrimethylammonium bromide
$CuSO_4$	Copper (II) sulfate
cv	Cultivar
DDS	DNA Double Strand
DH	Double Haploid
DIRS	Dictyostelium Intermediate Repeat Sequence
DNA	Deoxyribonucleic Acid
DRs	Direct Repeats
Ds	Dissociation transposon
DSBs	DNA Double Strand Breaks
FDA	Food and Drug Administration
GB	Giga base pair
GBS	Genotyping-by-Sequencing
GDNA	Genome DNA
GFP	Green Fluorescent Protein
GUS	β-glucuronidase
GWAS	Genome Wide Association study
HE-TAIL-	High-throughput Thermal asymmetric interlaced PCR
hph	Hygromycin-B-phosphotransferase
hpt	Hygromycin phosphotransferase
IAA	Indole Acetic Acid
IPCR	Inverse PCR
ISSR	Inter-Simple Sequence Repeat

JHCI	Joint Health Claims Initiative
LD	Linkage Disequilibrium
LINEs	Long Interspersed Nuclear Elements
LTRs	Long Terminal Retrotransposons
MCs	Minimal Gene Cassettes
MMT	Million metric tons (*i.e.*, Gg)
MS	Mineral Salts of (Murashige and Skoog)
N; K; P	Nitrogen; Potassium; Phosphor
NAA	Naphthalene Acetic Acid
NCBI	National Center for Biotechnology Information
NDA	Nutrition and Allergies
NOS	Nopaline Synthase
OCPs	Oat Callus Pieces
OCS	Octopine Synthase
ORF	Open reading frame
OT	Oat Transformant
OTP	Oat transposant
PCR	Polymerase chain reaction
PEG	Polyethylene Glycol
PLEs	Penelope-like elements
QTL	Quantitative Trait Locus
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RILs	Recombinant Inbred Lines
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Reverse Transcriptase
SBC	Stakman-Borlaug Center
SCAR	Sequence Characterized Amplified Region
SINEs	Short Interspersed Nuclear Elements
SNPs	single-nucleotide polymorphisms
SSR	Simple Sequence Repeat
T-DNA	transferred DNA
TEs	Transposable Elements
Ti Plasmid	Tumor-Inducing Plasmid
TILLING	Targeting Induced Local Lesions in Genomes
TIR	Terminal Inverted Repeats
TSDs	Target Site Duplications
uidA	β-glucuronidase
USDA	United States Department of Agriculture
uv	Ultraviolet
V(D)J	variable (V), diversity (D), and joining (J) gene segments
WDV	Wheat Dwarf Virus

YFP	Yellow Fluorescent Protein
β-Glucan	Beta Glucan
μE	microeinstein

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PREFACE

This thesis is written and designed as a manuscript-based form according to the "Guidelines Concerning Thesis Preparation" of McGill University.
All thesis components are original work. Chapter III is designed as a manuscript format for publication. The candidate is the primary author of this manuscript, while the contribution of co-authors can be found below.

CONTRIBUTION OF CO-AUTHORS

All experiments were designed by Jaswinder Singh and Mohannad Mahmoud. The experiments and analyses were performed by Mohannad Mahmoud. Rajvinder Kaur contributed to the transformation work. Mohannad Mahmoud and Jaswinder Singh wrote the manuscript.

CHAPTER I: GENERAL INTRODUCTION

1.1. Introduction

As cereals crops are the world's primary food source, their progressive improvement is crucial. Oat (*Avena sativa* L.) is one of the important cereal species cultivated worldwide (Boczkowska and Onyśk 2016)

Oats have multifunctional uses as human food and livestock feed, in addition to its role in crop rotations for soil conservation (Stewart and McDougall 2014; Mukumbareza et al. 2016). In 2018 the global oat production was 23.55. Russia and Canada being the main producers at 4.80 and 3.45 respectively. Canada is the main exporter of oats (USDA, 2018). Attention towards the cultivated oat has risen given the crop's adaptability, its multiple uses, and, mainly, its nutritional health benefits. Hence, in terms of consumption, oat is the fourth-most important cereal, after wheat (*Triticum æstivum* L.), rice (*Oryza sativa* L.), and corn (*Zea mays* L.) (Nazareno et al. 2018). However, oat still lags behind in terms of studies on its genetics and genomics, thereby impeding the further improvement of this important cereal. Recently developed empirical and computational genomic sequencing techniques can provide precious sequences and comparative genetic data. However, in large hexaploid genomes such as that of oats, gene sequencing and linking of genotypes to phenotypes remains a challenge. Thus, the development of new genetic mutants in oat is imperative and essential to explore the oat genome. Among several functional genomic techniques, transposon tagging systems, particularly the maize *Ac/Ds* transposons have been successfully applied in heterologous species. The *Ac/Ds* system has mainly been used to identify genes, promoters, and enhancers by knocking out or tagging activation techniques (Long et al. 1993; McElroy et al. 1997; Chin et al. 1999; Gorbunova and Levy 2000; Kolesnik et al. 2004; Ipek et al. 2006; Liu et al. 2007; Lazarow and Lütticke 2009; Carter et al. 2013; Xuan et al. 2016; Kim et al. 2018). Using this system, several important functional genes have been identified in heterologous species, including dicots such as tomato (*Solanum lycopersicum* L.) (van der Biezen et al. 1996), and monocots including barley (*Hordeum vulgare* L.) (Tripathi et al. 2018; Anwar et al. 2018b), and rice (Margis-Pinheiro et al. 2005; Manimaran et al. 2017)

Therefore, applying this transposable element-based system in oat offers the potential to explore this genome, and hence improve oat breeding programs. Accordingly, the present project was designed to build a transposons-based functional genomic resource for the oat genome, identify novel genes associated with dietary fiber, oil content, disease-resistance, and ultimately improve agronomic traits.

1.2. Hypotheses

1. Genetic transformation employing the maize *Ac/Ds* transposons system is feasible in oat.
2. A heterologous *Ac/Ds* transposons system can be a useful approach for gene tagging in oat.

1.3. Objectives

1.1.1 Study 1: Introduction of *Ac/Ds* elements into oat Genome

Aim 1: Observation of transient activity of *Ac/Ds* transposons in oat

Aim 2: Genetic transformation of oat genome for the development of stable *Ac/Ds* lines using different *Ac/Ds* constructs.

Aim 3: Confirmation of transgenic *Ac/Ds* activity in oat.

1.1.2 Study 2: Development of an *Ac/Ds* tagging population for the identification of *Ds* mutants.

Aim 1: Observation of *Ds* transposon activity in the oat genome through molecular analysis.

Aim 2: Generation of *Ds*-flanking sequences and their alternative insertion sites.

CHAPTER II

Literature Review

2.1. Oat and its significance

Oat *(Avena sativa* L.*)* is one of the main cultivated cereals worldwide. This multipurpose crop is mainly used in livestock feeds (Forsberg and Shands 1989). However, given its nutritional properties, its popularity as a healthy human food has dramatically increased (Kaur and Singh 2017). This growth in human consumption is mainly associated with the water-soluble fiber *β*-Glucan, a linear polysaccharide consisting of β- (1, 3) and β- (1, 4) linkages. *β*-Glucan is present in the wall of the oat aleurone and sub-aleurone endosperm cells (Ren et al. 2003; Kale et al. 2013) and plays a pivotal role in lowering plasma cholesterol levels, and thus in reducing the risk of coronary heart disease (Liu et al. 2016; Pomeroy et al. 2001). The health benefits of consuming the oat β-Glucan were recognized and confirmed by the U.S. Food and Drug Administration (FDA), the UK Joint Health Claims Initiative (JHCI), Health Canada, and the EU Nutrition and Allergies (NDA) in 1997, 2004, 2007, and 2010, respectively (Gorash et al. 2017). In addition to high-quality protein that exceeds 15% in modern oat cultivars (Sunilkumar et al. 2017; Zwer 2010), oat also has unique antioxidants such as avenanthramides (Collins 1989), vitamin E (Gutierrez-Gonzalez et al. 2013), and B complex including biotin, folic acid, thiamin, and pantothenic acid (Butt et al. 2008). Furthermore, oat contains many important minerals such as Mg, Se, Mn, Fe, Ca, Cu, and Zn (Welch 1995).

The world's oat production is mostly concentrated in Europe and the Americas. The Russian Federation is the largest producer of oat grain followed by Canada with about 4.8 and 3.45 mega metric tons (MMT) of production, with cultivated areas of 2.75 and 1.0 million hectares, respectively. In 2018, approximately 47% of the total Canadian oat production (*i.e.*, roughly 1.6 MMT) was exported, mainly to the USA (USDA, Statistics Canada 2018), making Canada the greatest oat exporter worldwide (Figure. 2.1, 2.2).

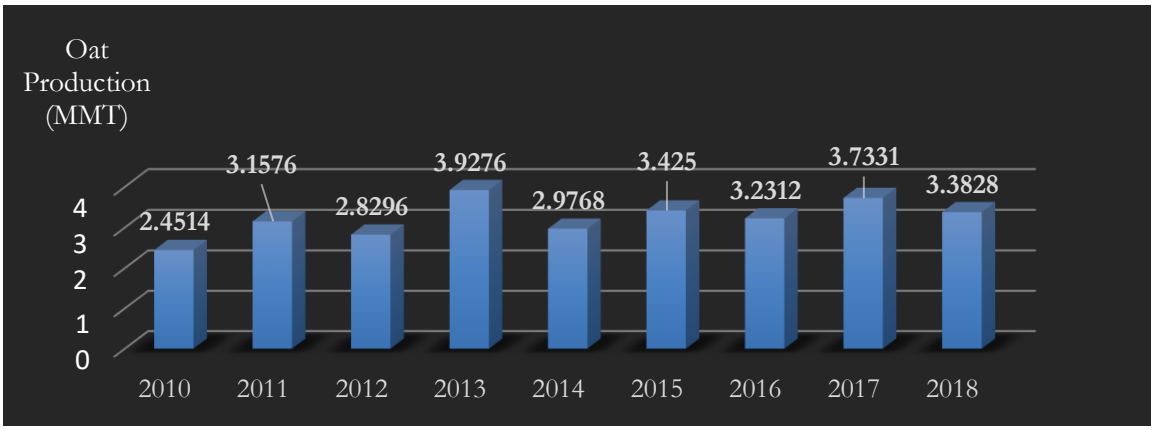


Figure 2.1 Canadian oat production, 2010-2018.
Statistics Canada, 2018 https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=3210035901

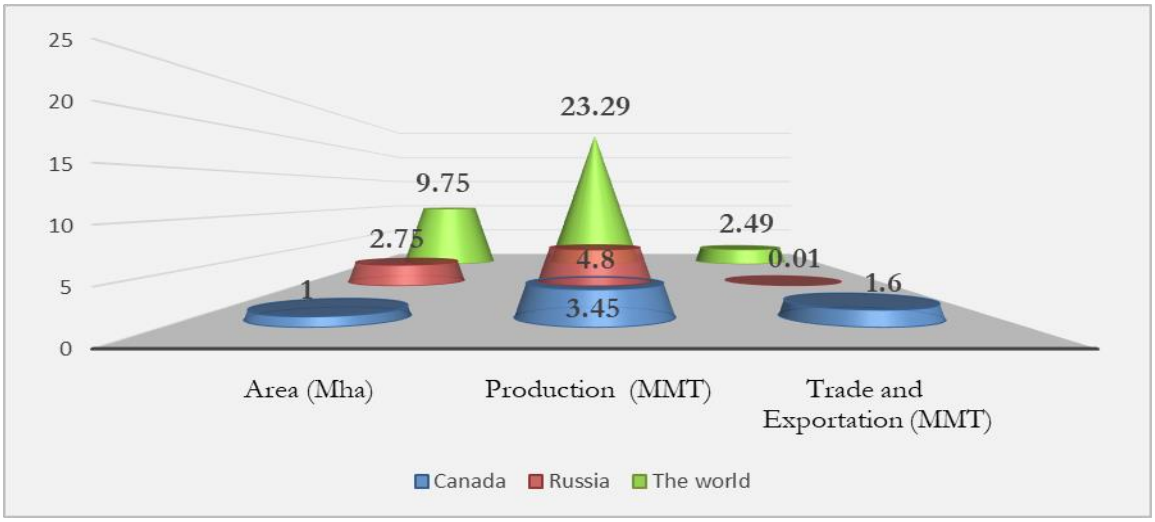


Figure 2.2 Oat hectarage, production and exportation in the world, Russia and Canada.
United States Department of Agriculture, 2018.
http://usda.mannlib.cornell.edu/usda/current/worldag-production/worldag-production-09-12-2018.pdf

Cultivation of oat dates to two thousand years ago (Murphy and Hoffman 1992). The major genetic diversity of oat is found in the Middle East, the Mediterranean and the Himalayas. Although its origin remains unclear, it was likely restricted to the cold and wet climatic conditions of the northern zones of western Europe (Zwer 2010). Compared to the other cereals, oat is widely adapted to growing in slightly cool-wet low precipitation environments with low-fertility soils (Buerstmayr et al. 2007; Ren et al. 2007). There are two basic economical phenotypic categories of oats: hulled (covered) and hulless (naked). Both categories belong mainly to the hexaploid forms of *Avena sativa* (Gazal et al. 2014; Menon et al. 2016). Oat hull is a non-starch carbohydrate consisting mainly of cellulose, in addition

to largely insoluble dietary fiber (Knudsen 1997; Coenen et al. 2006). However, the nutrient density of the oat grain is negatively correlated with the presence of the hull (Burrows et al. 2011). As hulless oats have a higher grain quality than hulled oats, they are preferred by the industry (Ren et al. 2007). For example, Winkler et al., (2018) reported on the economic potential using three naked oat varieties ('AC Gwen', 'Streaker', 'Paul') to replace wheat and corn in organic poultry diets. However, Givens et al. (2000) showed hulled oat cultivars ('Image' and 'Gerald') to have a greater β-glucan content than naked cultivars ('Pendragon' and 'Kynon'). The naked oat varieties also have a lower grain yield than hulled varieties, which limits its cultivation (Peltonen-Sainio 1997). The low grain yield of naked oat is mainly linked to its lower rate of seedling emergence, which is associated with the high rates of fungal infection and mechanical damage to its seeds. This sensitivity is due to the absence of palea and lemma in naked oat, and thus, leaving the caryopsis unprotected against external conditions (Valentine and Hale 1990).

The long global history of oat breeding programs has generated a remarkable number of both hulled and hulless oat cultivars. Despite the great value of oat and its products, the development of new genetic and genomic resources for its improvement has lagged. Recently launched, the Oat Global (https://oatglobal.umn.edu) organization seeks to establish "a public-private partnership committed to improving resilience, quality, and value of oat by coordinating precompetitive research, breeding, and extension on a global scale." The Oat Global partnership includes participants from governments, academia, and industry, in addition to other oat stakeholder and commodity groups. This partnership is led by the Stakman-Borlaug Center (SBC) for Sustainable Plant Health, associated with the University of Minnesota. Moreover, the Oat Genome project (http://avenagenome.org/) has developed a program which focuses on exploring hexaploid oat (*Avena sativa*) (ACD genome) by sequencing its diploid references including *A. eriantha* (CC-genome), and *A. atlantica* (AA-genome), using Pacific Biosciences (Pac Bio) sequencing. Despite these global efforts, oat hexaploid genomic and genetic knowledge is still limited, and thus, extensive investigations are required for the functional identification of its whole genome.

2.2. Oat breeding and genetics

Crop domestication over 10000 years ago was one of mankind's greatest cultural achievements (Buckler et al. 2001). However, this long-term selection process narrowed the gene pool of cultivated crops. The limitation of this pool drew attention to wild ancestors and landraces as treasures of

germplasm diversity (Newton et al. 2011; Pingali 2015). Such genomic evolutionary information provides crucial data that can be used in crop breeding programs. Plant breeding is the fundamental science that is used to improve and obtain modern varieties. According to Brown and Forsberg (1987), the classical cultivar development program includes five major stages: selection of parents according to the features desired, crossing these parents, testing repeatedly for the required traits, inbreeding, and selection among the subsequent generations, and finally, reduplication, conservation, and distribution of seed stocks. Any breeding program for oat cultivars should consider several essential issues, which including the producers' needs in a specific environmental area, the main intended usage of the crop for food or feed, and the deficiency in varieties to fulfill the needs (reviewed in Stuthman, 1995).

Thirty species have been identified under the genus *Avena*, and classified in a series of ploidy levels; diploids, tetraploids, and hexaploids (Baum 1977; Ladizinsky 1998). This series represents a wide variation in genome sizes (from 4.1 to 12.8 GB) of *Avena* species (Yan et al. 2016b). Global efforts have been taken to protect these valuable resources, and around 131,000 *Avena* accessions have been collected from different countries. Oat collections are the eighth largest germplasm collection in the world. Canada holds the largest collections, followed by the United States, and Russia with roughly 40000, 22000, and 12000 accessions, respectively (Singh and Upadhyaya 2015). The rest of these collections are deposited in other countries (Figure 2.3). Among these accessions, up to 75000 are classified as accessions of the cultivated oat species. These species include predominately, the common oat (*Avena sativa*) with (95%) of the collections, followed by red oat *(A.byzantina)*, Bristle oat (*A.strigosa)*, and Ethiopian oat *(A. abbyssinica)* (Singh and Upadhyaya 2015).

The common oat (*Avena sativa*) mainly covers the commercial oat cultivars worldwide (Bunte 2013), divided into the hullless and hulled oats (Gorash et al. 2017), which differ in a single main gene (*N-1*) responsible for the loss of the hull. This gene shows incomplete dominance and interacts with three modifying genes (*N-2, N3,* and *N4*) (Marshall and Shaner 1992). A completely hulless phenotype is only induced if homozygous dominant alleles are presented in the *N-1, N-2,* and *N3* loci (Jenkins and Hanson 1976). However, modern naked oat cultivars show a range of 1-15% hull content (Lawes and Boland 1974; Welch 1995), as hulless phenotypic expression varies according to environmental conditions, particularly temperature and humidity (Lawes and Boland 1974; Marshall and Shaner 1992).

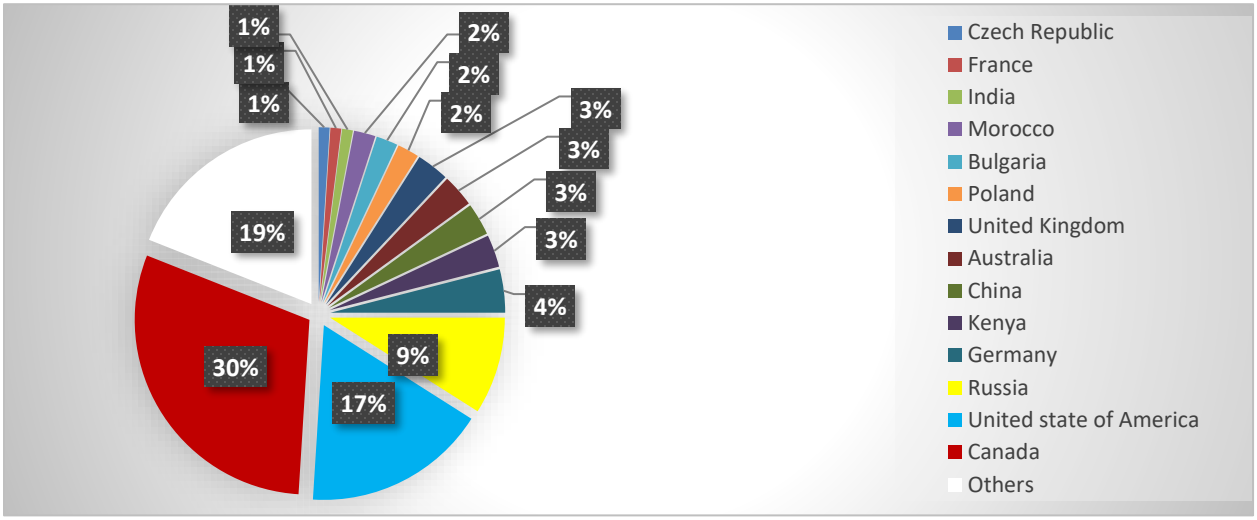


Figure 2.3 Global *Avena* collections by countries contribution (Singh and Upadhyaya 2015)

The common oat (*Avena sativa*) has a large (12.8 GB) repetitive hexaploid genome (2n = 6x =42) with a basic chromosomal number of seven (Yan et al. 2016b). The oat complex genome consists of three genomes (A, C and D), which are assumed to have originated from diploids possessing A, C, and D genomes. Interestingly, no D or B diploid genomes have been identified in diploid oat species (Ladizinsky 2012). Presumably, a CD genome tetraploid was initially generated, followed by hybridization to an (A) genome diploid, in which chromosomal doubling took place at each stage of the hybridization (Yan et al. 2016a). Recent studies have assumed that the A genome of the common oat is likely to have been derived from A and C group of diploid oat species which are *A. longiglumis* (Yan et al. 2016a), and *A. canariensis* (Chew et al. 2016) respectively. Moreover, three tetraploid *Avena* species (*A. insularis, A. murphyi, and A. maroccana*), which contain the ancestral D genome, are the potential donors of the *A. sativa* C and D genomes. However, due to the genomic similarity, the D genome is considered as a variant of the A genome (Yan et al. 2016a).

The remarkable efforts of oat breeding programs have led to the improvement of several qualitative and quantitative features, as well as tolerance and resistance to a number of abiotic and biotic stresses. These traits and their genetic sources are reviewed elsewhere (Brown and Patterson 1992; Huang et al. 2014). A wide range of the *Avena* accessions have been recruited in these breeding programs (Kiviharju 2016). For instance, Okoń et al. (2016) introduced the importance of *A. maroccana, A. murphyi, and A. sterilis* L. genotypes as sources of powdery mildew resistance.

Moreover, a great number of oat genetic markers are provided for oat adaptation, yield, lodging and quality traits, abiotic and biotic resistance (Achleitner et al. 2008; Newell et al. 2012; Montilla-Bascón et al. 2015; Winkler et al. 2016; Tumino et al. 2017; Gnocato et al. 2018; Zhao et al. 2018; Rispail et al. 2018). Recent genotyping by sequencing techniques have generated a large number of single-nucleotide polymorphisms (SNPs) and sequence information, including 4975 SNPs (Tinker et al. 2014), and 88199 nucleotide sequences (NCBI, 2018). This information is available in several databases, including GrainGenes (https://wheat.pw.usda.gov oat /ggpages/maps.shtml#oats), the National Center for Biotechnology Information, NCBI (https://www.ncbi.nlm.nih.gov/gquery/?term=oat), Oat Global (https://oatglobal.org/), and the oat genome project http://avenagenome.org/. This genetic information is highly valuable for discovering and introducing genes important for oat improvement (Huang et al. 2014).

2.3. Molecular and functional genomic studies in oat

The first QTL (quantitative trait loci) molecular linkage map in hexaploid oat was generated in 1995 by O'Donoughue and his colleagues. They used a population of recombinant inbred lines (RILs) derived from a cross between *A. sativa L.* (cv 'Ogle'), and *A. byzantina* C. Koch (cv 'Kanota'). As a result, they mapped of 561 loci (O'Donoughue et al. 1995; Wight et al. 2003). In order to saturate the map further, several mapping populations and molecular markers, including Simple Sequence Repeat (SSR), Inter-Simple Sequence Repeat (ISSR), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Sequence Characterized Amplified Region (SCAR), Random Amplified Polymorphic DNA (RAPD), and Single Nucleotide Polymorphism (SNPs) were used (Jin et al. 2000; Groh et al. 2001; Wight et al. 2003; De Koeyer et al. 2004; Barbosa et al. 2006; Tanhuanpää et al. 2006) and a new genetic map with 625 DNA markers was updated in 2008 (Tanhuanpää et al. 2008).

The first physically-anchored linkage map of hexaploid oat was developed by Oliver et al. (2013), including 985 SNPs and 68, previously published, markers consisting of 21 linkage groups covering a distance of 1838.8 centimorgans (cM). Further markers of hexaploid oat were reported by Tinker et al. (2014), in which an array of 4975 SNPs was generated. Also, Song et al. (2015) constructed a linkage map of naked hexaploid oat, including 208 SSRs resolving 22 linkage groups. Moreover, the Genome-Wide Association Study (GWAS) was introduced as an alternative approach for the detection of the quantitative trait locus (QTL) in oat (Newell et al. 2011). In this approach, both the linkage disequilibrium (LD) decay and the markers' density were included. Also, Genotyping-by-Sequencing

(GBS) system is a robust and low-cost tool for genomic and plant breeding studies (Elshire et al. 2011). This technique was applied for genomic exploration in oat, enabling to place 45,117 loci on the consensus genetic map of oat (*A. sativa*) (Huang et al. 2014). Recently, 4657 accessions of the cultivated common oat were used for haplotype-based GBS analysis. As a result, 70000 loci were added to the oat consensus map (Bekele et al. 2018). However, a limited number of association analysis studies based on marker-linked traits were reported in oat. These traits included β-Glucan concentration (Asoro et al. 2013), spikelet number (Pellizzaro et al. 2016), heading date and plant height (Esvelt Klos et al. 2016; Zimmer et al. 2018), disease resistance (Montilla-Bascón et al. 2015; Gnanesh et al. 2015; Sunstrum et al. 2018), and adaptation (Sunstrum et al. 2018).

Through global collaborative efforts, a total of 99878 mapped markers have been placed on the oat consequence map (Bekele et al. 2018), an important milestone for molecular breeding and gene discovery analysis in oat genetics and breeding. However, compared to the other main cereals, the genetic study of oat has lagged behind that of other cereals. This has been largely due to the oat genome's complexity, limitations in research funding, and the lack of aneuploid and mutant stocks (Gazal et al. 2014; Gorash et al. 2017).

2.4. Oat tissue culture

2.4.1. Explants and callus induction

The regenerative capacity of the plant cell through its ability to divide, differentiate and become a complete plantlet using in-vitro media set the stage for plant tissue culture. Lack of success in obtaining plantlet regeneration in the first such experiment some 180 years ago (Schwann 1839), was linked to the lack of knowledge respecting growth substances (Schulze 2007). The central term of the tissue culture science, callus, represents undifferentiated tissue that originally consisted of parenchymatous cells. Several factors play critical roles in the ability of an explant passing through the callus stage to regenerate a complete plantlet, including: (*i*) the chemical components of the *in vitro* growth media used, particularly, plant growth regulators and minerals, (*ii*) tissue culture environmental conditions including the temperature, humidity and light, (*iii*) the donor genotype, and (*iv*) the callus-derived explant type used (Trigiano and Gray 2016).

Over the years, several studies took place to detect the responses of various explants to different tissue culture procedures and medium components, and thereby discover those best suited for the plant regeneration ability. Identification of auxins (*e.g.*, 3-indole acetic acid — IAA) in tobacco (*Nicotiana*

tabacum L.) plants helped initiate plant tissue culture system. These growth regulators include auxins and cytokinins (*e.g.*, 6-furfuryleaminopurine) (Skoog and Miller 1957). MS medium (Murashige and Skoog 1962) is the most successful media used for plant regeneration. Until 1980, there were only a few reports of successful in-vitro plant regeneration in monocots, particularly in the grass family (Vasil 1987). However, using tissues that contain undifferentiated and meristematic cells as explants, and adding 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin, provided the basis to regenerate the main cereal species (Morrish et al. 1987). These tissues include portions of mesocotyl (Torne et al. 1980; Jelaska et al. 1984), leaf sheath (Chen et al. 1995; Gless et al. 1998a; Ahmadabadi et al. 2007), shoot meristems (Zhang et al. 1996; Sticklen and Oraby 2005; Pilahome et al. 2014), immature embryos (Eissa et al. 2017; Dong and Chen 2017), along with mature embryos and seeds (Özgen et al. 1998; Cho et al. 1999; Chauhan and Khurana 2017; Gatphoh et al. 2018). For example, up to 60% regeneration frequency was obtained using oat leaf bases as explants (Chen et al. 1995).

Among various explants, the immature embryo, given its high ability to regenerate *in vitro*, remains the predominant main explant used in the cereal transformation-based research (Gasparis 2017). However, the supply of explants from immature embryos is limited by time and fully dependent on the donor plants' life cycle. Moreover, the possibility of higher somaclonal variation due to the prolonged regeneration of these embryos under tissue culture conditions has affected the frequency of its use (Gasparis 2017).

Callus regeneration capacity over a longer period is very important for plant transformation applications. This continuity of the ability to regenerate was reported for the first time in oat callus, after frequent sub-culturing of callus derived from immature embryos (Cummings et al. 1976). Several factors affect this capacity, particularly, the explants, media components, oat genotype, and tissue selection during subculture (Rines et al. 1992). In the genetic transformation of oat, several explants (tissues) have been used: immature embryos (Torbert et al. 1995; Perret et al. 2003), leaf base segments (Gless et al. 1998b), mature embryos (Torbert et al. 1998; Cho et al. 1999), and shoot apical meristems (Zhang et al. 1999; Cho et al. 2003). Using mature seeds as explants for callus induction, and a biolistic system as a gene delivery method, Cho et al. (1999) established a highly reproducible and efficient *in vitro* system for oat callus induction, regeneration, and transformation.

2.4.2. Regeneration medium

The major components of regeneration medium include macronutrients, micronutrients, a source of carbohydrates, growth regulators, vitamins, nitrogen supplements and amino acids, as well as solidifying agents in the case of solid or semi-solid media (Trigiano and Gray 2016). Moreover, some undefined supplements, such as banana homogenate, and organic acids have also been used in some circumstances (George et al. 2008). As in the other main cereals, adding 2,4-D to the medium was the key factor for initiating the oat callus successfully (Webster 1966). 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), a synthetic auxin, has also been successfully used for oat callus induction (Nabors et al. 1982). According to Rines et al. (1992), the oat calli were cultured on solid media (6-10 g L^{-1} agar) containing the mineral salts (MS) of Murashige and Skoog (1962), 2-5 mg L^{-1} of (2,4-D), 20-40 g L^{-1} of sucrose, and the vitamins used for tobacco callus initiation by Linsamier and Skoog (1965). B5 media, developed by Gambrog et al. (1968), had similar positive effects as MS on oat callus regeneration (Rines and McCoy 1981). Adding specific growth regulators to MS media, such as 0.2 mg L^{-1} of 6-benzyleamoniopurine (BAP), and 2mg L^{-1} naphthaleneacetic acid (NAA) further improved callus regeneration efficiency (Rines and Luke 1985). In contrast, hormone-free media accelerated the formation of both shoots and roots of oat embryogenic tissue callus (Bregitzer et al. 1989).

Cho et al. (1999) applied three induction mediums on calli derived from mature oat seeds. Calli pieces served as targets in the biolistic transformation process. These media contain the same components as the previous regeneration media, but concentrations of 2,4-D, BAP (6-Benzylaminopurine), and $CuSO_4$ were different. Using DB3 medium that bore greater concentrations of $CuSO_4$ and BAP, but a lesser concentration of 2,4-D, refined the regeneration ability. They also used another medium containing similar components as DB3, but with equimolar amounts of sorbitol and mannitol, as osmotic media. A treatment of the calli with osmotic media was required before they could be subjected to the bombardment step. This led to the successful development of a highly efficient delivery of particles into the cells. In this approach, all the selected events retain the ability of regeneration, and up to 26% transformations efficiency have been reported (Cho et al. 1999).

2.4.3. Selection processes and antibiotics

The selection process is critical as an initial confirmation step of the putative transgenic candidates. The principle behind this technique is to identify transgenic calli based on specific genes included in the plasmid cassette. These genes provide resistance to antibiotics, herbicides, or other stresses, in

which are imposed in growth media or by generated fluorescence, facilitating the selection process. There are many strategies described in the literature for the selection of transgenic calli. These include selectable and reporter genes (reviewed in Rosellini, 2012). The most common selectable genes in plant transformation include *Bar*, *hpt*, *hph, NPTII*, and *PMI* genes, while the main reporter genes include *GUS*, *GFP*, *RFP*, and *YFP*.

For instance, some selectable genes provide resistance against specific antibiotics, such as hygromycin phosphotransferase (*hpt*), and neomycin phosphotransferase (*NPTII*) genes (van den Elzen et al. 1985), that confer resistance to hygromycin and kanamycin, respectively. The *Bar* gene, providing resistance to the BASTA/IGNITE (Glufosinate) herbicide is used as a selectable marker (Murakami et al. 1986). Moreover, the gene which encodes phosphomannose isomerase (*PMI*) in *Escherichia coli* has also been used as a selection marker, for the first time in a transformation-based study in sugar beet (*Beta vulgaris* L.) (Joersbo et al. 1998). In that study, sucrose in the tissue culture medium for sugar beet was either totally or partially replaced by mannose. The phosphomannose isomerase enzyme can convert D-mannose into D-fructose-6-P, which can be metabolized if the *PMI* gene is expressed. Accordingly, transgenic calli bearing this gene can survive on the media supported by mannose rather than sucrose (Rosellini 2012). Further selectable genes can be expressed differently, such as *GFP* (green fluorescent protein), and YFP (yellow fluorescent protein) genes. These genes were derived from the jellyfish (*Aequorea victoria*), the *GFP* and *YFP* proteins when present in living cells or tissues exhibit a shiny green or yellow fluorescence when exposed to light of a specific wavelength (Ormo et al. 1996): 396-475 nm in the case of *GFP*, and 514 nm in the case of *YFP* (Voss et al. 2013). Such gene expression can indicate not only transient events but also to the activity of this gene in the transgenic organisms and their progeny (Zimmer 2002). Furthermore, the β-*glucuronidase* gene (*uidA*, or *GUS*) has also been used as a reporter gene in plant transformation systems. The gene was first isolated from *Escherichia coli* (Novel et al., 1974) and developed as a reporter gene by others (Jefferson et al. 1987). Several features make the *GUS* gene one of the best reporter systems for plant transformation, including: its stability (Jefferson et al. 1987) and high sensitivity to β-glucuronidase activity that can be detected by histochemical, spectrophotometric, and fluorometric techniques (Cervera 2005). In oat transformation systems, several selectable markers have been used including *uidA*, *Bar*, *nptII*, *GFP*, *hph*, and *hpt* (Torbert et al., 1995; Gless et al., 1998a; Torbert et al., 1998; Cho et al., 1999; Zhang et al., 1999; Kaeppler et al., 2000; Kuai et al., 2001; Maqbool et al., 2009; (Dattgonde et al., 2019). For example, a plasmid carrying both *Bar* and *uidA* genes was successfully delivered into calli derived from

the spring oat cultivar (GAF-30/Park) using the gene gun technique (Somers et al., 1992). This study reported a transformation frequency of 34% and led to the first transgenic oat plants. Moreover, Gasparis et al., (2008) used both Kanamycin resistance (*nptII*) and *GUS* genes as selectable markers for the selection of transformed oat calli grown from two hulled ('Bajka', and 'Slawko'), and one hulless ('Akt') cultivars. Using an *Agrobacterium*-mediated transformation technique, these genes were introduced into oat (cv 'Bajka') calli, and transformation frequency of up to 12.3% was achieved. A recent study in which an *Agrobacterium*-mediated transformation technique was employed yielded a transformation frequency of up to 21.85% and 25% using the hygromycin-B-phosphotransferase (*hph*) and *GUS* genes, respectively (Dattgonde et al. 2019).

2.5. The genetic transformation in plants

The applications which allow the introduction, overexpression, or inactivation of specific genes in plants provide powerful functional genomic techniques (Sessa et al. 1994). Genetic transformation mechanisms are considered evolutionary methods because of their numerous experimental advantages, including: (*i*) their contributions of creating and optimizing novel qualitative and quantitative characteristics, particularly, in modern varieties, and (*ii*) introducing or modifying crop resistance against several insects, viruses, herbicides, and post-harvest deterioration. These achievements were difficult to obtain using traditional breeding programs (Gatehouse et al. 1992; Hinchee et al. 1993; Theologis 1994; Shah et al. 1995; Yuan et al. 2017).

Transforming a novel foreign gene into a host plant using a polyethylene glycol technique was first achieved and reported for tobacco (Paszkowski et al. 1984). An extensive array of further transformation experiments have been conducted, through which up to 120 species have successfully been transformed. These species include many economically important crops, trees, vegetables, fruit, medicinal and pasture plants, and ornamentals (Birch 1997). Several methods for gene delivery are applied in plants, including electroporation, polyethylene glycol (PEG), lipofection, microinjection, sonication, silicon carbon fiber, ultraviolet laser microbeam (uv), viral vector mediated, *in planta*, *Agrobacterium*-mediated transformation, and particle bombardment using the Gene gun (Biolistic) (reviewed in Keshavareddy et al., 2018). Among these methods, *Agrobacterium*-mediated and biolistic are those most commonly and successfully applied for transformation systems in monocots species.

2.5.1. Agrobacterium-mediated genetic transformation

Agrobacterium tumefaciens.has been widely used to generate transgenic plants, due to its capacity to transfer a part of its DNA, known as T-DNA (transferred DNA) via the Ti plasmid, into a host plant genome (Sheng and Citovsky, 1996). This plasmid was identified in bacterial crown gall tumors and is considered an essential tool for its inducibility (Schilperoort, 1967; Van Larebeke, 1974; Zaenen, 1974). This transformation system includes five fundamental stages, including: (*i*) bacterial virulence system induction, (*ii*) T-DNA complex generation, (*iii*) T-DNA transferring from *Agrobacterium* to the host cell nucleus, (*iv*) T-DNA integration into the plant genome, and (*v*) T-DNA gene expression (Gelvin, 2010; Pitzschke and Hirt, 2010; Tzfira and Citovsky, 2006; Ziemienowicz, 2001). These steps generally include tissue culture and regeneration steps. However, it can also be coupled with *in-planta* transformation processes rather than passing callus induction stages. Several modifications of the *in-planta* methods have been introduced, including agroinfiltration, vacuum infiltration, spraying, sonication, floral drop, and dip (Ratanasut et al. 2017).

The *Agrobacterium* transformation system has several beneficial features: (*i*) the occurrence of single or few exogenic copies, (*ii*) good fertility, and (*iii*) stable expression of transgenes. These advantages render this a preferred method of genetic transformation for a large portion of plant species. Several main cereal crops were subjected to this transformation technique, such as wheat (*Triticum aestivum*) (Cheng et al., 1997; Supartana et al., 2006), rice (*Oryza sativa*) (Supartana et al., 2005), Barley (*Hordeum vulgare*) (Wan and Lemaux 1994 ; Tingay et al. 1997; Kumlehn et al. 2006), maize (*Zea mays*) (Chumakov et al., 2006; Ishida et al., 1996; Mamontova et al., 2010; Anand et al. 2018; Anand et al. 2019), rye (*Secale cereal* L, sorghum (*Sorghum bicolor* (L.)(Zhao et al., 2000; Gao et al., 2005; Nguyen et al., 2007; Che et al. 2018). Gasparis et al., (2008) generated the first successful transformed oat using an *Agrobacterium*-mediated method. They used two husked ('Slawko', and 'Bajka'), and one naked ('Akt') cultivar. Leaf base segments, and/or immature embryos were used as oat explants. *GUS* expression analysis was performed in both T_0 and T_1 generations for confirmation of the transgenic status. As a result, the highest transformation frequency (12.3%) was achieved using immature embryos of 'Bajka,' using Kanamycin (nptII gene) antibiotic selection. Using the same method, Oraby and Ahmad (2012) successfully introduced the Arabidopsis *CBF3* gene, into oat calli using *(hpt)* hygromycin selection, providing the transgenic oat lines with a higher tolerance to salinity stress. The *Agro*-transformation rate in oat varies. In a recent study, *Agrobacterium*-mediated transformation of calli derived from leaf base and mature embryo explants of oat cultivar 'JO-1' was enhanced by using vacuum infiltration

and sonication (Dattgonde et al., 2019). Using *hph* and *GUS* selectable markers they were able to achieve transformation frequencies of up to 19.04%. Their maximum transformation efficiency was achieved by applying a 72-hour dark-vacuum treatment on leaf-base-derived calli (Dattgonde et al., 2019).

Several factors affect transformation frequency including: genotypes (cultivars), explants, and the features of plasmids used in transformation (Oraby and Ahmad 2012; Gasparis 2017). Despite the successful *Agrobacterium* transformation of cereals, efficiency remains low, likely because cereals are not natural hosts of *Agrobacterium tumefaciens* (Vasil 2005), which leads to a slower wound response, and thus a low or complete absence of the activation of virulence genes. Studies suggest that embryogenic cells could improve wound response, and improvements in this method for cereals could be achieved (Komari and Kubo 1999). Regardless, this method is still one of the main transformation mechanisms in cereal crops.

2.5.2. The biolistic genetic transformation approach

The biolistic or Gene Gun transformation plant transformation method was described in 1987 by the scientists E.D. Wolf, N.K. Allen, and John C. Sanford (Sanford et al. 1987), then further refined by Sautter et al. (1991). This system was created as an alternative protoplast transformation method, particularly for recalcitrant cereals. Biolistic is a physical process based on the bombardment of the target tissues by gold or tungsten microparticles coated with foreign DNA or the gene of interest (Jefferson et al. 1987; Christou 1992; McAllister 2000). For a successful transformation through bombardment, several parameters must be considered: the DNA concentration, cell rupture pressure, distance between the target tissue and the microcarrier, microcarrier particle size, as well as the plant tissue type and its ability to regenerate after the transformation process (Vain et al. 1993; Lemaux et al. 1996; Cho et al. 1998b; Able et al. 2001).

This method has some drawbacks, such as introducing multiple transgene copies and hitting random intracellular targets. These disadvantages may lead to complex transgenic locations resulting in inappropriate segregation, causing loss of fertility and inducing gene silencing (Pawlowski and Somers 1996; Kohli et al. 1998; Choi et al. 2000; Darbani et al. 2008; Gasparis 2017). In contrast, Singh et al. (2006) obtained a single or a low number of transformations while maintaining fertility, in the generation advance of transgenic barley. Similarly, a range of 16.1 to 73.5% single copy insertion was achieved in bread wheat (cv 'Gladius') using the Biolistic system (Ismagul et al. 2018)

Several important benefits make Biolistic the method of choice for wide-range genetic transformation objectives and targets:

- The flexibility of being able to apply the system to the tissues and cells of a wide range of organisms, including recalcitrant species (Altpeter et al. 2005; Gao and Nielsen 2013).
- The ability to introduce multiple genes in a single simultaneous step (Vidal et al. 2003).
- The ability to apply circular or linear plasmids, or minimal gene cassettes (MCs) as efficiently as transferring whole plasmids (Keshavareddy et al., 2018). These MCs are generated by removing all the vector backbones before particle loading, thereby not involving the plasmid in the host genome (Vidal et al. 2006).
- The capacity to integrate new genetic editing techniques, such as CRISPR/Cas9, in addition to its application in generating marker-free genetically modified plants (Liu et al. 2013; Zhang et al. 2016).

Given these features, this system has seen dynamic and successful use in the transformation process of many cereal cultivars. Wheat was the first inheritable transgenic plant obtained by introducing the *Bar* gene using the biolistic system (Vasil et al. 1992). Application of this system was further reported in wheat (Ismagul et al. 2018; Hamada et al. 2018; Tian et al. 2019), rice (Li et al. 1993; Feng et al. 2017; Mortazavi and Zohrabi 2018), maize (Zhang et al. 2002; Sidorov et al. 2016), barley (Yao et al. 1997; Wahara et al. 2017), and sorghum (Zhu et al. 1998; Grootboom et al. 2010; Belide et al. 2017). Using this system, transformation frequencies of up to 50% were achieved for bombarded cereal explants (Li et al. 1993).

Using the biolistic method, calli derived from the spring oat cultivar 'GAF-30/Park' were bombarded with a plasmid carrying both *uidA* and *Bar* genes, yielding a transformation frequency of 34%, and thereby generating the first transgenic oat plants (Somers et al. 1992). However, of these transgenic events, only one plantlet was fertile. Numerous experiments have been conducted to optimize several transformation factors including genotype, explants and selectable markers (Cho et al. 1999; Gless et al. 1998a; Kaeppler et al. 2000; Kuai et al. 2001; Maqbool et al. 2009; Torbert et al. 1998; Torbert et al. 1995; Zhang et al. 1999). In these studies, calli were derived from different explants, such as leaf bases, immature and mature embryos, and mature seeds. Several oat cultivars were used, including 'Park,' 'Garry,' 'Jumbo,' 'Melys,' 'Ogle,' 'Pacer,' 'Prairie,' and 'Fuchs.' Among these cultivars, one spring oat cultivar ('Park') has been commonly used in oat transformation-based studies. While the

transformation frequency reported in these studies, has ranged from 0 to 83%, this frequency has not been calculated properly. For example, Cho et al. (1999) claimed a stable transformation efficiency of 26%, based on the fertile transgenic oat lines generated, whereas Maqbool et al. (2009) who reported an up to 83% transformation efficiency calculated this rate based on the number of calli pieces which passed the selection process. The high transformation frequency claimed in the latter study is skewed as several factors were not considered: regeneration ability, fertility, and transformation stability. However, compared to other transformation systems, the biolistic technique remains the most efficient technique for stable genetic transformation in oat.

2.6. Functional genomics strategies

The replacement of landraces by modern cultivars, has led, over the long term, to a considerable narrowing of the allelic diversity of cultivated cereals. This narrowing has been examined and reported in maize (Roussel et al. 2004; Warburton et al. 2008), wheat (Reif et al. 2005), and the cultivated oat (Coffman 1977; Fu et al. 2003). Thus, an understanding of genetic variation in oat and a knowledge of its genome are essential components for future breeding programs. These goals can be achieved by applying efficient mutagenesis approaches, allowing a deeper understanding of the genome to be achieved. Several mutagenesis-based systems have contributed to the functional identification of unknown genetic sequences, including: T-DNA insertion, transposon tagging, map-based cloning, RNA interference (RNAi), and Targeting Induced Local Lesions in Genomes (TILLING) methods (Alonso and Ecker 2006; Parry et al. 2009; Sikora et al. 2011; Li et al. 2011; Watanabe et al. 2011; Singh et al. 2012). Identification of the key genes linked to desirable plant traits in crop species was the main objective of these approaches. These systems aim to improve the crop' quantitative and qualitative traits, and heighten its resistance or tolerance to biotic and abiotic stresses. By 1995, at least 1700 mutant varieties had been created in 154 plant species (Maluszynski et al. 1995), while by 2004 this number had risen to over 2500 (Ahloowalia et al. 2004). In oat, several mutant populations were reported to have been developed using these techniques. For instance, a population of 2550 mutants of spring oat (cv. 'SW Belinda') was constructed by Chawade and his colleagues (2010), using a TILLING technique. Generating this population led to the identification of two genes *AsCslF6*, and *AsPAL1*, key factors in the pathways of lignin and β-glucan biosynthesis (Chawade et al. 2010). Despite the low-cost of the TALLING technique and its achievements, it has several disadvantages: a low-mutagenic induction efficiency, and the need for highly skilled specialists to implement it (Khan et al. 2018).

The discovery of transposable elements and their mobility through the genome introduced made these elements excellent tools to use in an efficient mutagenesis approach. This system relies on the transformation of these elements into host plant genomes and the exploration of their transposition events and their alternative insertion sites. These steps are followed by linking unique genotypes to their phenotypes or *vice versa* (Lazarow et al. 2013).

2.7. Transposable Elements (TEs)

The transposable Elements (TEs) are fragments of DNA that can insert themselves into novel host chromosomal locations throughout the genome. Some of these elements can duplicate themselves, and increase their copies during the process of transposition (Feschotte et al. 2002). Their transposition and replication capacity enables these genetic factors to colonize almost all the genomes that have been sequenced up to date. This colonization includes eukaryotic, prokaryotic and even archaeal domains (Zaratiegui 2017). For instance, the percentage of these elements in the human genome is at least 45% (Lander et al. 2001). In most grass species genomes, TEs colonize 50-80% of the genome (Sanmiguel and Bennetzen 1998; Cantu et al. 2010). Moreover, their genomic load has been found in more than 80% of maize and wheat genomes (Jamilloux et al. 2017). These elements were discovered in maize by the eminent geneticist and Nobel Laureate Barbara McClintock in the 1940s. Different color patterns in maize kernels were linked to transposable elements (also known as jumping genes),subsequently named as *Activator* (*Ac*) and *Dissociation* (*Ds*) elements (McClintock 1948).

There are two basic classes of transposable elements based on the mediator (RNA, or DNA) and the transposition mechanism. The two classes (class I and class II) have been further classified into subclasses and orders, based on their enzymology and characteristics of the transposition mechanism.

2.7.1. Class I (Retrotransposons)

The class I transposons are also known as Retrotransposons and use an RNA strand intermediate for their transposition (Finnegan 1989; Biémont 2010). The RNA strand is first transcribed from a genomic DNA, then reverse-transcribed into a DNA double strand (DDS) by the TE-encoded reverse transcriptase (RT) protein. The resulting DDS element is then reinserted into the host genome, though a mechanism known as copy and paste. As a result, within one complete duplication cycle, one copy of the retrotransposon is generated (SanMiguel et al. 1996; Kumar and Bennetzen 1999).

Class I (TEs) can be divided into five basic orders: long terminal retrotransposons (LTRs), long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), Penelope-like elements (PLEs), and Dictyostelium Intermediate Repeat Sequence (DIRS) (Wicker et al. 2007).

Among Class I orders, LTR retrotransposons are the most widespread order in plant genomes (Gaut and Ross-Ibarra 2008). This order consists of several families, however, few of them are found in cereals, *e.g.*, *BARE1* in barley (Vicient et al. 2000), *Angela* in wheat (Wicker et al. 2001), *Retrosor6* in sorghum (Peterson et al. 2002), and *Opie* in maize (SanMiguel et al. 1998). Several studies have reported the activation of these elements under extreme abiotic or biotic stress conditions (Grandbastien 1998). For example, activation of *Tos17* in rice has been observed under tissue culture conditions (Hirochika et al. 1996). Likewise, activation of *OARE1* transposons in the oat genome was spotted under UV light, and after fungal inoculation (Kimura et al. 2001). The interaction between stresses and retrotransposon activity indicates their evolutionary importance with respect to subtle shifts in environmental conditions.

2.7.2. Class II (DNA Transposons)

The transposable elements related to this class, use DNA fragments in their transposition mechanism. These elements are classified into two subclasses based on the number of the DNA strand cleaved during the transposition process.

2.7.2.1. Transposons subclass I

These transposons can transpose by a cut and paste mechanism, where an initial break in the double strand DNA (DSBs) is induced, and the cleaved DNA fragments are transferred into alternative chromosomal sites (Schulman and Wicker, 2013).

This subclass of TEs has been classified into two orders: terminal inverted repeats (TIR) and Crypton. The TIR order elements can increase their copies over the transposition process, mediated by transposase enzymes during the chromosome replication. The TIR translocation process occurs from a chromosomal locus that has already been duplicated, to another one ahead of the replication fork. As a result of the transposition mechanism, the process will yield an additional copy of one of the daughter chromosomes (Greenblatt and Brink 1962; Schulman and Wicker 2013). The TIR order contains nine superfamilies, which vary mainly in length. Most of these families contain autonomous and non-autonomous elements. The autonomous elements encode different transposase enzymes. These transposase proteins share similar motifs, including a narrowing of the gap between the DDE motifs glutamate and aspartate residues which leads to the formation of a catalytic center. This structure enables the transposase to excise DNA transposons, and likely to reinsert them into the novel sites (Baker and Luo 1994; Doak et al. 1994; Keith et al. 2008)

In contrast, Crypton order elements can encode a tyrosine recombinase enzyme, as can some retrotransposons, but they lack the reverse transcriptase enzyme, so these elements are also transposed by DNA cut and paste mechanisms. Interestingly, they lack terminal inverted repeats; however, DSBs are generated likely through its transposition mechanism (Goodwin et al. 2003).

2.7.2.2. Transposons subclass II

These elements transpose by copy and paste mechanisms, whereby the replacement of one strand of DNA occurs rather than cleavage of the double strands. Similarly to the first subclass, subclass II is also classified into two orders, commonly known as Helitron and Maverick. Helitron uses the rolling-circle mechanism for their transposition (Kapitonov and Jurka 2001) whereas, Maverick transposes through the excision, extrachromosomal replication, and reinsertion steps (Kapitonov and Jurka 2006).

2.8. DNA transposons in the plant kingdom

Transposable elements dominate significant portions of plant kingdom genomes. The main superfamilies of plant DNA transposons include *Helitron, Tc1–Mariner, Mutator, P, PIF– Harbinger, CACTA*, and *hAT*. These superfamilies have various characteristics, and structures as classified by Wicker et al. (2007) and updated and reviewed by Zhao et al. (2016). An example of these superfamilies is shown in (Table 2.1).

Moreover, high frequencies of multiple copies of silent transposons have been found in plant genomes (Wicker et al. 2007). The DNA transposons' mobilization likely occurs because of special circumstances, such as biotic or abiotic stresses, as observed in the retrotransposons (Makarevitch et al. 2015). This mobilization activity plays a critical role in genetic expression, regulation, and reshaping of the genomes during the evolution of plant species (McClintock 1993; Biémont 2010). In oat, knowledge about TEs is limited. Assembly of the smallest oat chromosome, 18D revealed a large proportion of TEs in the genome, including 80% LTR retrotransposons, 16% DNA transposons, and 3% non-(LTR) retrotransposons (Luo et al. 2012). Moreover, both pAs17 and OARE-1 LTR-retrotransposons were identified in a cultivated hexaploid oat, where, respectively, 24000 copies (Linares et al. 1999), and 10000 copies (Kimura et al. 2001) of each were estimated to exist. No comprehensive estimation of TEs in the hexaploid oat genome is currently available.

Table 2.1: Classification, and a brief structural feature of the main super-families of DNA transposable elements in plants. Features include the presence and length of the terminal inverted repeats (TIRs) in the transposable element, the target site duplications (TSDs), and the presence of the DDE motif in the proteins.

	Classification		TIRs	TSDs	Transposase
Subclas	Order	Superfamily			DDE (motif)
I	TIR	*Tc1–Mariner*	11-120	Varied	Present
		P	31 bp*	8	None
		PIF–	14-60 bp	3	Present
		CACTA	12-28 bp	2-3	None
		hAT	5-22 bp	8	Present
		Mutator	0-800 bp	9-11	Present
II	Helitro	*Helitron*	none	0	None. This element does not encode transposase

* (O'Hare and Rubin 1983)

2.8.1 Maize *Activator/Dissociation* transposons

Activator (*Ac*) and *Dissociation* (*Ds*) are the first transposable elements discovered and characterized by Dr. Barbara McClintock (McClintock 1948). Analyzing chromosomal breakage in maize using light microscopy, McClintock found a highly frequent breakage in one strand of chromosome 9 at a specific locus. Through genetic analysis of maize plants and their progeny she discerned two genetic elements. One of them, located in the same breakage locus, was named (*Ds*) *Dissociation*, while the other played a catalytic role for the same chromosomal breakage, and was thus named (*Ac*) activator. Barbara McClintock received the Nobel Prize in 1984 for this discovery (Comfort et al. 2001).

Ac/Ds elements are class II TEs which belong to the *hAT* family, TIR order, and subclass I. These elements can transpose via DNA fragments by substitutional translocations using a cut and paste mechanism. The autonomous *Ac* contains a specific sequence encoding *Ac*-transposase (*AcTpase*). This enzyme identifies the 3' and 5' terminal repeats of both *Ac* and *Ds* elements, and then catalyzes the transposition to alternative chromosomal sites (Kunze and Starlinger 1989). The non-autonomous *Ds* element resembles most of the incomplete sequences of the *Ac* element (Fedoroff et al. 1983). However, the *Ds* element lacks the intact (*AcTpase*) encoding gene. The absence of this enzyme in the *Ds* element prevents its ability to transpose unless an *AcTpase* source is provided.

2.8.1.1. Maize *Activator (Ac)* transposon structure

With a simple structure of 4,564 bp, the Maize *Ac* autonomous element bears two 11bp terminal inverted repeat sequences (TIRs), and 250–300 nucleotide subterminal regions at both ends (Coupland et al. 1988; Varagona and Wessler 1990). These sequences are essential to its *Ac*-transposition mechanism. The *Ac* element also contains a transcription unit for a single 3.5 Kb messenger RNA. This unit consists of five exons encoding the 807 amino acids transposase enzyme (*AcTpase*) (Kunze et al. 1987). The maize *Ac* element has a major initiation transcription site for mRNA, at location 334 from the 5' end, in addition to several sites between 304 and 364 nt. At the 3'-end, it has terminator of 265 nt. The *AcTpase* (*ATG)* start codon is located at position 988, while the *TGA* stop codon is at position 4063 (Figure 2.4) (reviewed in Lazarow et al. (2013)). The *Ac* promoter is considered a low-activity promoter due to lack of TATA and CAAT boxes (Kunze and Weil 2002).

2.8.1.2. Maize *Dissociation (Ds)* transposon classification and structure

The maize Dissociation element (*Ds*) has two main structures. The simple *Ds* element is generated from deletion of an *Ac* element and shares two 11bp TIRs but differs in its internal sequence. A second type of *Ds* element is more complex and bears multiple ends in alternate orientations (Lazarow et al. 2013).

The maize *Ds* elements are classified into three types: *Ds-del, Ds1,* and *Ds2*. The *Ds-del* elements have sequences of variable length derived from the maize *Ac* element, arrived at by altering the transposase encoding gene (Conrad et al. 2007). The *Ds1* elements are small (~400 bp); however, they bear the same 11bp TIRs, in addition to a few subterminal hexametric repeats of *Ac* elements (Peacock et al. 1984). The third type, *Ds2* elements, are over 1 kb in length and share comprehensive homology with the *Ac* element, especially 200bp sequences at both terminals (Merckelbach et al. 1986). In addition to these three types of *Ds* elements, 900bp *Ds*-related sequences were identified in maize line B73 by Du et al., (2011). These new elements have been found to be somewhat related to the previously described three *Ds* types. These elements have been named as *Dsl 3, and Dsl 4* and classified as *Ds*-like elements as they also share two 11pb TIRs and are considered as part of the *hAT* transposon family. However, they lack the highly conserved *C* terminus of *hAT* family transposons. *Dsl 3* elements also contain sequences derived from two exons (2 and 3) of the transposase encoding gene. In contrast, *Dsl 4* elements only share a 30pb sequence of the *Ac* element at either end (Du et al. 2011).

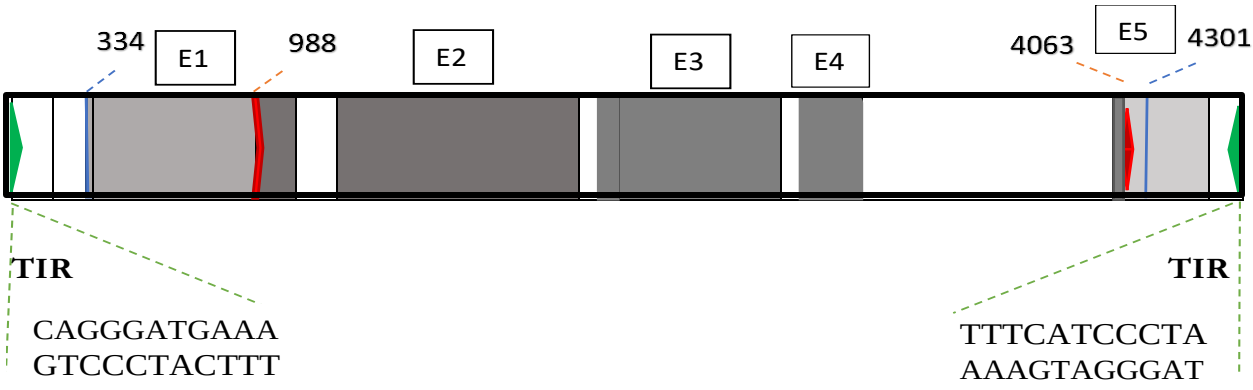


Figure 2.4 The maize *Activator* (*Ac*) element structure containing five exons E (1-5), the major site of transcription at position 334, *ATG* Start Codon at position 988, *TGA* Stop codon at position 4063, The Polyadenylation site is at position 4301, and two 11bp terminal inverted repeats (TIR) occur at both ends.

2.8.1.3. The *Ac/Ds* cut and paste mechanism

Ac/Ds elements, as in all transposons related to the *hAT* family, share the same transposition mechanism. This mechanism includes enucleation of *Ac/Ds* elements from their original location into another chromosomal site. The insertion of *Ds* in the new locus causes 8 pb duplications on both sides of the insertion site. These repeats are known as direct repeats (DRs) or target site duplications (TSDs).

Further alterations may occur at the insertion site if a *Ds* transposition event happens again. This alteration is known as the transposition footprint. Both intact 11bp TIRs and 8pb (DRs) are key to *Ac/Ds* elements' transposition ability (Singh et al. 2006). This footprint may include large deletions or insertions. For instance, Page et al. (2004) reported a large chromosomal deletion, adjacent to a *Ds* donor site, ranging from 64 to 104 kb, in an *Arabidopsis* plant model.

In most maize *Ac/Ds* deletions, the transposition process follows a hairpin model. This hairpin-mediated transposition consists of several steps. The *AcTpase* enzyme catalysis the cleavage of each DNA strand, one base pair distal to the 5' ends of the *Ac / Ds* elements, leaving a free 3'-hydroxyl group. A hairpin is formed by nucleophilic attack of the phosphodiester bonds at the 3'-end, by the free hydroxyl group resulting from the cleavage, and the transposons are released (reviewed in Lazarow et al. 2013).

The cut and paste transposition mechanism involves a hairpin formation, which is, hypothetically, similar to the hairpin formed during the V(D)J recombination process mediated by DDE recombinase

enzymes (Lu et al. 2006). *Ac*Tpase and recombinase enzymes share a highly conserved tryptophan residue. The *AcTpase* protein bears a tryptophan residue at position 464, likely contributing to the formation of a flipped-out base in the hairpin center causing the hairpin to open. This flipped-out base causes one strand-cleavage and leads to a cut in the center of the hairpin. The repair machinery rejoins and fills in the single-strand gap (Lazarow et al. 2013).

Reinsertion of the excision *Ac*/*Ds* transposons occurs at alternative chromosomal sites. This reinsertion is likely initiated by the integration of the free hydroxyl groups in the transposon ends. In both upstream and downstream regions of the insertion site 8bp repeats (TSDs) are generated. The DSRs are not specific and generate any combination of 8bp repeats (Du et al., 2011). The *Ac*/*Ds* machinery of this breakage, hairpin formation, repair, and reinsertion of transposons in the plant genome has yet to be fully explored. However, the activity of the DDE center apparently catalyzes the transposition mechanism (Zhou et al. 2004). The transposition of the *Ac* element, from its donor site, can occur either into replicated or non-replicated sites. Such transpositions occurs particularly during or soon after the cell cycle's S (interphase) stage (Chen et al. 1992).

2.8.1.4. *Ac* transposase enzyme *(Ac*Tpase*)* activity

The dramatic reduction of *Ac* transposase expression observed in advanced generations of barley (Kaeppler et al. 2000) likely occurs because of an epigenetic silencing mechanism, *e.g.*, genomic cytosine methylation. For example, increased DNA methylation activity has been observed in dedifferentiated cell cultures from *Ac* insertion lines in rice (Kohli et al. 2004). Several studies also reported the decrease or loss of *Ds* transposition through the line of progeny of transgenic rice (Izawa et al. 1997; Chin et al. 1999; Nakagawa et al. 2000; Upadhyaya et al. 2002). This loss of the transposition activity is likely linked to a declining expression of the transposase.

In contrast, there was no loss of *Ds* transposition ability in advanced generations of barley *Ds* insertion lines. This stability was explained by the observation of unmethylated *Ds* terminals (Singh et al. 2006). Further maintenance of transposition activity, in the advanced generations, was also reported in *Ac*/*Ds* transgenic rice (Szeverenyi et al. 2006).

2.8.1.5. Extrachromosomal reactivation of modified *Ds* element

The extrachromosomal activity of the *Ds* element can be induced by the transient expression of *Ac* transposase (*Ac*Tpase). This activity leads to the transposition of *Ds* element, without the integration of the *Ac* coding gene in the host genome (Laufs et al. 1990; Becker et al. 1992; Shen et al. 1998). By applying a simple transient assay, McElroy et al. (1997) showed evidence for the extrachromosomal activity of the maize *Ac* element in the barley genome. They transformed two *Ac*/*Ds* constructs into barley scutellar tissues. The *Ac* construct served as a source

of *Ac* transposase, while the *Ds* construct bore the *uidA* (β-glucuronidase) gene, interrupted by the *Ds* element. The expression of *uidA* was obtained only when *Ds* excision occurred under catalysis of the *Ac*Tpase protein. As a result, several *GUS* foci were observed, confirming the transient activity of *Ac*Tpase in these scutellar tissues. Correspondingly, *Ds* excision events under the transient expression of *Ac*Tpase were obtained in wheat, by Takumi et al. (1999). They bombarded wheat lines, harboring the *Ac*Tpase gene, with an *uidA* interrupted *Ds* construct, and noted the appearance of blue color upon applying a *GUS* histochemical assay**.** Further successful in somatic transposition activity of the *Ds* element was obtained in rice by Upadhyaya et al. (2006). Using an *Agrobacterium*-mediated method Rice plants harboring the *Ds* element were transformed by an *Ac* transposase encoding element fused with the GFP selection marker. The ensuing transient expression of *Ac* transposase led to a *Ds* transposition frequency of 9-13%. Moreover, transient expression of *Ac*Tpase was also reported in *Hieracium aurantiacum* L. (Weld et al. 2002), bell pepper (*Capsicum annuum* L.) (Kim et al. 2004a), tobacco (Fitzmaurice et al. 1992), and sorghum (Verma et al. 2011). This *Ac*-transient expression based-*Ds* excision technique provides a precious tool for generating new instant first generation stable *Ds* lines due to the loss of *Ac*Tpase expression. Also, it can accelerate the reactivation process of the *Ac/Ds* system, since no crossing of mature *Ac/Ds* plants is needed (Weld et al. 2002).

2.8.1.6. Maize *Ac/Ds* transposons as an efficient mutagenesis system in plant species

Several transposable elements have been used for functional genomic studies in cereals including: *En/spm, Mu, Tos17* and *Ac/Ds* (Hirochika 2001; Settles 2009; Fladung 2016). Predominantly chosen as an efficient alternative to T-DNA mutagenic systems, the *Ac/Ds* transposon-mediated approach has several advantages (Qu et al. 2008), including:

i. The bias of *Ds* towards genetic regions, *e.g.*, more than 70 % of transposition events in maize, and transgenic rice and barley occurred in/or close to genetic regions (Cowperthwaite et al. 2002; Kolesnik et al. 2004; Singh et al. 2006). Thus, this transposition machinery can generate a wide range of tagged alleles, which are essential for the efficient characterization of genes and their function.

ii. High-frequency remobilization through the progeny, such that generating initial transgenic *Ac/Ds* lines is enough to develop a significant number of different *Ds* insertion events (*Ds* Transposants) (Singh et al. 2006; Szeverenyi et al. 2006). This remobilization activity can overcome the transformation difficulty in the recalcitrant cereal crops.

iii. Insertion of the *Ds* element simultaneously with the *Ac* activity element can generate stable mutations. This stability is due to the segregation of *Ac* element from *Ds* in the progeny (Springer 2000).

iv. Can be used for induction of random overexpression mutations. Generating this kind of mutants can overcome obstacles of redundancy in the genome (Ayliffe et al. 2007; Ito et al. 2005; Peterson and Zhang 2013; Singh et al. 2006)

Genetic redundancy has been considered as a major hurdle in any functional genomic system, because it may prevent the emergence of phenotypes in the mutants (Nakazawa et al. 2003). This situation occurs because of the overlapping functions of several genes. For example, due to the redundancy of *LOB* genes, no visible phenotype change was observed after insertion of T-DNA in one *Arabidopsis LOB* gene (Shuai et al. 2002). Moreover, pleiotropy could also mask the phenotype and thus obstruct the precise identification of a gene-phenotype correlation (Springer 2000). Because of its unique features, the *Ac/Ds* system has been widely used as a saturation mutagenesis approach for functional genomic studies (Walbot 2000; Fladung and Polak 2012).

Tobacco was the first successful heterologous model for the introduction of the maize *Ac* transposon into its genome (Baker et al. 1986). This system was then successfully used for functional genomic studies of other dicots such as *Arabidopsis* (Bancroft and Dean 1993; Kuromori et al. 2004; Ito et al. 2005), potato (*Solanum tuberosum* L.) (Lu et al. 2014; Knapp et al. 1988; Lu et al. 2015), strawberry (Veilleux et al. 2012; Lu et al. 2014), tomato (Meissner et al. 2000; Carter et al. 2013; Cooley et al. 1996), soybean [*Glycine max* (L.) Merr.] (Mathieu et al. 2009; Sandhu and Bhattacharyya 2017), broccoli (*Brassica oleracea* L.) (Mckenzie and Dale 2004), lettuce (*Lactuca sativa* L.) (Yang et al. 1993), and carrot (*Daucus carota* L.) (Van Sluys et al. 1987; Ipek et al. 2006).

In cereals, the maize *Ac* transposable element was first introduced into rice protoplasts, using an electroporation technique (Izawa et al. 1991). In this study, the maize *Ac*-element-interrupted *hph* gene was used, such that the excision of *Ac* from its construct led to the expression of the *hph* gene, and resistance of the antibiotic hygromycin. The *Ac* transposition activity in transformed rice calli was confirmed by hygromycin phenotyping, and Southern blotting analysis. This efficient experiment was followed with successful transposon-based functional genomic research in several cereal crops, including rice (Izawa et al. 1997; Enoki et al. 1999; Chin et al. 1999; Kim et al. 2004b; Kolesnik et al. 2004; Qu et al. 2008; Guiderdoni and Gantet 2012), barley (Koprek et al. 2000; Scholz et al. 2001; McElroy et al. 1997; Zhao et al. 2006; Lazarow and Lütticke 2009), wheat cells (Takumi et al. 1999),

sorghum (Pushpalatha 2013; Verma et al. 2011), and *Brachypodium distachyon* (L.). (Wu et al. 2019; Bragg et al. 2012). The two component *Ac/Ds* system is more efficient than the insertion of an autonomous one component *Ac* system. According to Koprek et.al. (2000), up to 47% transposition efficiency was observed in F2 progeny. This F2 population was generated by self-pollination of F1 lines carrying both the *Ds*Ubi-*Bar* element and *Ac*Tpase. However, Singh et al. (2006) further refining the *Ac/Ds* system in barley examined *Ac*Tpase and *Ds* activity for four generations. They found that a crossing between *Ac*Tpase, and single *Ds* copy lines led to secondary, tertiary, and quaternary transposition frequencies of 16.9 %, 17.1 %, 16.4 %, respectively. These results provide evidence of the stability of *Ac*Tpase expression, and the ability to remobilize *Ds* activity through several generations. Furthermore, Ayliffe et al. (2007) reported *Ds* transposition frequencies of up to 50% in barley. This *Ds* remobilization was obtained by crossing lines carrying *Ac*Tpase with lines carrying the *Ds* activation tagging element.

The *Ac/Ds* mutagenic system has led to the observation of more than 1,500 *Ds* mutants in maize that extended through all 10 maize chromosomes (Vollbrecht et al. 2010). Comparatively, in rice, 20,000 *Ds* insertional mutants were obtained, of which 800 have improved our knowledge of several traits associated with abiotic stresses and the grain yield. These mutants can be employed in rice breeding programs (Jiang et al. 2007a; Jiang et al. 2018). A further 115,000 *Ds* insertion mutants were created by the Functional Genomics program in Korea, and 462 mutants were subjected to phenotypic characterizations (Kim et al. 2018). In 2014 and 2018, respectively, populations of 70 and 61 homozygous *Ds* tagged barley lines were developed and released by the USDA-ARS (Brown et al. 2014; Bregitzer et al. 2018).

2.8.1.7. *Ac/Ds* approach as a functional genomic approach for gene tagging

Maize *Ac/Ds* transposons were successfully applied to a number of plant species in a reverse genetics approach for gene identification. Several genes were isolated and functionally identified. For instance, this transposon system once active in tomato (Yoder et al. 1988), was used to isolate: (*i*) *CF-9*, the resistance gene for the fungal plant pathogen (*Cladosporium fulvum*)(Jones et al. 1994), (*ii*)the *Dicer Like DCL* gene, required for palisade cell morphogenesis, (*iii*) the *FEEBLY* gene involved in chloroplast development (Keddie et al. 1996), which also has an important function in plant development and metabolism (van der Biezen et al. 1996), (*iv*) the *SDL-1* gene, which encodes a highly conserved protein with a plastid targeting motif in *Nicotiana plumbaginifoliaI* Viv. (Majira et al. 2002). Recently, Anwer et al (2018) and Tripathi et al (2018) used a *Ds*-tagged micro RNA gene (miR172), created by Brown and

Bregitzer (2011), to explore the regulatory function of miR172 in floral development. As a result of this study, they revealed the role of miR172 in controlling cleistogamous flowering (Anwar et al. 2018a) and the role of the Squamosa-promoter binding-like (*SPL*) genes in flower architecture (Tripathi et al., 2018) in barley. In addition, a male sterility phenotype was obtained by *Ds* insertion into the myosin XI B gene (*osmyoXIB*) of rice (Jiang et al. 2007b). This infertile phenotype was observed only under short day conditions, while the mutant showed a regular fertility phenotype under long day conditions. Accordingly, the *osmyoXIB* gene is linked to pollen development in a photoperiod-related manner.

Notwithstanding the successful introduction of this system into genomes of many cereal crops, no such system is currently available in *Avena* species. Accordingly, we applied this system, for the first time, in the common oat (*Avena sativa* L.). Developing such transposon-based resource in oat will provide a significant milestone in the area of oat functional genomics.

CONNECTING STATEMENT

Recent discoveries regarding oats' functional human nutritional health components (*e.g.*, soluble fiber β-glucan, and important avenanthramide antioxidants), and their ability to enhance the human immune system and optimize cholesterol and sugar levels in the blood, have given rise to a growing global interest in the common oat, and need for efficient breeding programs to improve current oat cultivars. Despite the impressive improvements in high throughput sequencing techniques, efforts towards sequencing the complex hexaploid oat genome are still lagging. Moreover, the limited genetic and genomic information regarding oats poses a serious challenge, hindering the improvement of oat's desirable traits. Accordingly, there is an urgent need to develop a large mutant tagging population for gene identification in oat. Unlike the T-DNA system previously introduced into the oat genome, the maize *Ac/Ds* transposon-based system has advanced features. In particular, a large *Ds* mutant population can be generated from a few transposons transgenic *Ac/Ds* lines. This feature is associated with the remobilization ability of the *Ds* element when it is cross-hybridized with an *Ac* transposase source. Such remobilization is very critical in cereal crops, where the transformation process is not routine. The Maize *Ac/Ds* transposon system has been successfully applied in several monocot and dicot species, including important cereal crops such as rice, barley, and wheat. These applications have led to the identification of several dominant genes, and their functions. We therefore hypothesize that the transformation of heterologous *Ac/Ds* into the oat genome is feasible and that such a system can be used efficiently as a mutagenesic approach for functional genetic identification in oat. The development of such a heterologous transposon resource in oat presents a significant potential milestone for the exploration of the hexaploid oat genome, and the identification of its genes and their functionality. Expansion of our research can develop further mutant populations for transposition event screening. Moreover, phenotypic mutants obtained can be genetically identified.

Chapter III is formatted as a publication manuscript. The co-authors are listed as follows: Mohannad Mahmoud, Rajvinder Kaur, Jaswinder Singh. Each co-author's contribution is described in detail, in the Preface.

CHAPTER III: GENETIC TRANSFORMATION OF OAT WITH MAIZE *AC/DS* ELEMENTS AND IDENTIFICATION OF *DS*-TAGGED LINES

3.1. Abstract

Grown worldwide, the common oat (*Avena sativa* L.) is an important cereal due to its multifunctional uses for animal feed and human food. Interest in this crop has grown substantially after the recent discovery of functional components linked to human health and well being in its seeds (Kaur and Singh 2017). Improving the desirable features and nutrients of current oat cultivars is imperative to meeting a growing global demand. Having one of the largest and most complex hexaploid genomes among cereals, oat has lagged behind other cereals in terms of genetic and genomic studies. This limitation impedes its further improvement. Nevertheless, the development of new genomic approaches in oat is essential for the characterization of its genome. Modified maize *Activator* (*Ac*) & *Dissociation* (*Ds*) transposon-based gene tagging systems has been successfully applied in many heterologous crop species, including barley, rice, and wheat. The progress of a *Ac/Ds*-mediated genomic approach, developed for the first time in oat is described. Using a biolistic gun, highly regenerative calli derived from mature oat seeds (cv 'Park') were genetically bombarded or co-bombarded with various *Ac/Ds* constructs. Molecular and biochemical analyses confirmed a total of 22 unique transformation events where *Ac* and/or *Ds* elements were successfully introduced into the oat genome. Transformation frequencies of up to 9.5% and 1.9% were achieved using screening with bialaphos and hygromycin, respectively. The *Ds* elements were further reactivated either through generation advance of oat lines containing both *Ac* and *Ds* elements or through transient transposase activity of *Ac* in calli obtained from *Ds* lines. This enabled and exploration of *Ds* mobilization, and thereby, the identification of novel *Ds* insertions. As a result, up to 16.9 %, and up to 15.9% transposition frequencies were observed through generation advance, and transient activity, respectively. Transposon activity was also confirmed by empty donor PCRs using primers flanking to both *Ds* ends. A very high excision frequency (74.6%) was noted in T0 using a transient activation assay. These results largely concurred with the 74% somatic *Ds* excision frequency, reported in an F2 rice population (Kolesnik et al. 2004). However, only half of these excision events (55%) were integrated in the rice genome. More investigations are required to explore whether our transient excision events are followed by *Ds* integration in the oat genome or not.

In order to identify *Ds*-tagged genomic regions and the structure of insertion/excision sites, *Ds* flanking sequences were generated. Using various approaches including inverse PCR, Tail-PCR and adapter-ligation PCR, 21 *Ds* flanking sequences were isolated. In most sequences, identity could not be retrieved. However, one *Ds* insertion was found to have occurred in the *Hordeum vulgare* GA 20-oxidase 3 (GA20ox3) gene (2e-13). Data about a mutant phenotype was also documented, although gene identity remains obscure. Success in the generation of transgenic *Ds*-transposon insertion lines will lead to the development of the first ever transposon-based functional genomic resource for oats. This transposon-tagging system will allow the identification of novel genes associated with desirable traits in hexaploid oats.

3.2. Introduction

Mainly produced in Europe (64.8%), and the Americas (27.5%), the cultivated oat is one of the most important forage and grain cereals in the world (USDA, 2019). Traditionally, oats were mainly used for livestock feed; however, interest in this crop has increased dramatically in recent times, particularly due to its recognition as a functional healthy food for human consumption (Kaur and Singh 2017), based on the nutritional and physiological features of several its components (Sterna et al. 2016). Amongst these, the water-soluble fiber polysaccharide *β*-Glucan, plays a pivotal role in lowering plasma cholesterol levels, thereby reducing coronary disease risk (Anderson and Siesel, 1990; Liu et al., 2016; Pomeroy et al., 2001). The health benefits of consuming oat β-Glucan have been recognized and confirmed by the FDA, the UK Joint Health Claims Initiative (JHCI), Health Canada, and the EU Nutrition and Allergies (NDA) (Gorash et al. 2017). Moreover, oats produced by modern cultivars contains a high-quality protein which exceeds 15% w/w of the seed content (Zwer, 2010; Rani, 2014; Sunilkumar et al, 2017), as well as unique antioxidants such as avenanthramides (Collins 1989), vitamin E (Gutierrez-Gonzalez et al., 203) and B complex, including biotin, folic acid, thiamin, and pantothenic acid (Butt et al., 2008). Furthermore, oat contains several important minerals such as Mg, Se, Mn, Fe, Ca, Cu and Zn (Welch, 1995).

Oat (Avena sativa) has a large and complex hexaploid genome (2n =6x=42), with a size of (12.8) GB, and a basic chromosomal number of (7) (Yan et al. 2016b). This genome consists of three nuclear genome sets (A, C, and D) (Ladizinsky, 2012), which are assumed to have originated through a series of evolutionary steps. Initially, a CD genome tetraploid was generated, which was followed by its hybridization to an A genome diploid. At each stage of these hybridizations chromosomal doubling occurred, thereby generating the oat genome's hexaploid status (Yan et al., 2016a). Three tetraploid

Avena species (*A. insularis*, *A. murphyi*, and *A. maroccana*) were the likely potential donors to the *A. sativa* CD genomes, while the A genome is likely derived from the Ac group of two diploid oat species: *A. canariensis* (Chew et al., 2016), and A. *longiglumis* (Yan et al., 2016a). Moreover, the recent evolutionary study of 25 *Avena* species indicated three diploid species (*A. canariensis, A. ventricosa*, and *A. longiglumis*), and two tetraploid species (*A. agadiriana,* and *A. insularis*), as potential sources of the cultivated oat's maternal genome (Fu 2018).

Published studies on DNA hybridization and manipulation, discrimination of genome size (Jannink and Gardner 2005; Oliver et al. 2010), and recent mapping strategies (Portyanko et al. 2001; Wight et al. 2003) are part of the long history of oat research. Past oat breeding programs improved the quality and quantity of the yield, and provided resistance to diseases such as stem and crown rust (McKinnon 1998). However, replacing local varieties with improved cultivars led to a reduced allelic diversity in the cultivated oat (Coffman 1977; Fu et al. 2003).

The first molecular linkage map of oat QTL (quantitative trait loci), with 561 loci, was generated in 1995, by O'Donoughue and colleagues. This map was further extended through an array of different techniques, including: Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), Simple Sequence Repeat (SSR), Inter-Simple Sequence Repeat (ISSR), Random Amplified Polymorphic DNA (RAPD), and Sequence Characterized Amplified Region (SCAR) (Jin et al. 2000; Groh et al. 2001; Wight et al. 2003; De Koeyer et al. 2004; Barbosa et al. 2006; Tanhuanpää et al. 2006). For example, a 625 DNA markers-based molecular linkage map was generated, by applying these techniques on the double haploid (DH) of the cultivated oat (Tanhuanpää et al. 2008). These markers include AFLPs, IRADPs, RADPs, SSR, ISSR, and Single Nucleotide Polymorphism (SNPs). The first physically-anchored linkage map of common hexaploid oat including 68 previously published markers and 985 novel SNPs was generated by Oliver et al. (2013). These markers were located in 21 linkage groups covering a distance of 1838.8 centimorgans (cM). Further identification of genetic markers in hexaploid oat was achieved by Tinker et al. (2014), by constructing an array of 4975 SNPs. Moreover, Song et al. (2015) developed a linkage map of naked hexaploid oat which included 208 SSR in 22 linkage groups, covering 2070.50 cM.

Besides the previous techniques, a Genome-Wide Association study (GWAS) was also applied for the identification of QTLs in common oat (Newell et al. 2011). In this system, both the marker density and linkage disequilibrium (LD) decay were included. In another study, a genotyping-by-sequencing

(GBS) system was introduced as a low-cost and robust tool for plant breeding and genomic studies (Elshire et al. 2011). Using this tool, a total of 45,117 loci were placed on the consensus genetic map of common oat (*Avena sativa*) (Huang et al. 2014). Recently, Bekele et al., (2018) applied haplotype-based GBS analysis on 4657 accessions of hexaploid oat, thereby adding a total of 70000 loci the common oat consensus map.

However, in terms of markers-linked traits, few association analysis-based investigations were applied in oat. These traits-based studies include the spikelet number (Pellizzaro et al. 2016), adaptation (Sunstrum et al. 2018), β-Glucan concentration (Asoro et al. 2013), and disease resistance (Montilla-Bascón et al. 2015; Gnanesh et al. 2015; Sunstrum et al. 2018). Global research efforts led to an improved oat consequence map with a total of 99878 markers (Bekele et al. 2018). While this map provides a valuable resource for future functional analysis, and can be used to improve oat breeding, several obstacles still hinder the effective improvement of oats: (*i*) narrowing of oat cultivars allelic diversity, due to replacing local varieties with improved cultivars, (*ii*) lack of the oat genetic and genomic data, linked to the considerable chromosomal rearrangements occurring in oats which disrupts the multicollinearity among the sub-genomes (Bennett and Smith 1976). Thus, the implementation of modern genomic/ biotechnological approaches, such as genetic transformation, RNAi, TILLING and gene editing are imperative if one is to build a functional genomic resource targeted to improving our understanding of the complex oat genome.

Among several functional genomic techniques, the *Ac/Ds* transposon-based approach has several advantages. In this system, few initial transformants are required to develop a large population of unique transposon tagging lines, which can be further reactivated on demand. Moreover, unlike the T-DNA insertional mutagenesis system, which requires frequent transformation, the transposon-based approach has been found to be a successful mutagenesis mechanism in species where transformation is not routine (Ito et al., 2002; Jeong et al., 2002; Ayliffe et al., 2007). Secondly, the unique *Ds* insertions can probably occur through either somatic tissues or gametes. In the case of somatic tissues insertions, transfer may occur in the same insertion event to multiple germline cells, then plants. In the case of insertions in the gametes, the resulting plants would contain unique various novel *Ds* insertion copies (Ayliffe and Pryor 2009). Thirdly, since *Ds* is a non-autonomous element, stable *Ds* mutants can be generated while an *Ac*Tpase source is segregated away. Such mutant lines can be used in oat breeding programs (Singh et al. 2006). Maize *Ac/Ds* system was successfully introduced in several dicots and monocots plant species, as a reverse genetic approach for gene

identification. For instance, Yoder et al., (1988) developed an *Ac/Ds* transgenic resource in the tomato genome. Using this resource, Jones et al., (1994) could identify the CF-9 gene involved in plant resistance to the fungal pathogen (*Cladosporium fulvum*).

Further genes were isolated using this technique in tomato, including: (*i*) the *DCL* gene, which is required for chloroplast development and palisade cell morphogenesis (Keddie et al., 1996), (*ii*) the *FEEBLY* gene, which has an important function in metabolism and plant development (van der Biezen et al., 1996), and (*iii*) the *SDL-1* gene, which encodes a highly conserved protein that plays a critical role in plastid biosynthesis in *Nicotiana plumbaginifolia* (Majira et al., 2002). Moreover, Anwar et al., (2018) revealed the role of miR172 gene in controlling cleistogamous flowering in barley, using a *Ds*-tagged micro RNA gene (miR172) mutant developed by Brown and Bregitzer (2011). Furthermore, a *Ds* insertion in rice myosin XI B gene (osmyoXIB) led to a male sterility phenotype only expressed under short-day conditions (Jiang et al., 2007b). This mutant revealed the important role of this gene in photoperiod-regulated pollen development.

Despite the successful application of *Ac/Ds* maize transposons system in cereals, no such report has been made in oat. Thus, introducing this system as an active functional tagging approach for the oat genome provides an extremely valuable tool to explore this genome, and uncover a myriad of beneficial oat genes.

3.3. Material and Methods:

3.3.1. Plant material and explants process

Mature seeds of spring oat cultivar ('Park') were surface-sterilized for 20 minutes in a 20% v/v bleach (5.25% sodium hypochlorite) solution, followed by three washes with sterile water. The sterilized seeds were put in MS (Murashige and Skoog 1962)-based callus generation media (Table 3.1), developed by Cho et al. (1998b), and maintained under dim light (16 h light, 10-30 μE), and temperature 24±1°C. The germinated roots and shoots were manually removed. After 3 weeks, high-quality green tissues containing embryogenic and nodular patterns were selected (Figure 3.1). These calluses were either subjected to the bombardment process or maintained in a different medium (DBC3), for 3-4 weeks.

Table 3.1: Different tissue culture media used during the transformation process

Component	Medium			
	Regeneration	Callus induction (DBC3)	Osmotic	Rooting
MS Salts	4.4 g/l	4.4 g/l	4.4 g/l	4.4 g/l
Maltose	-	30g/l	-	-
Sucrose	30g/l	-	-	30g/l
Casein hydrolysate	-	1g/l	1g/l	-
Myo-inositol	-	0.25 g/l	0.25 g/l	-
Proline	-	0.69 g/l	0.69 g/l	-
Thiamine HCl	1 mg/l	1 mg/l	1 mg/l	1 mg/l
Pyridoxine HCl	0.5 mg/l	-	-	0.5 mg/l
Nicotinic acid	0.5 mg/l	-	-	0.5 mg/l
$CuSO_4$	0.16 mg/l	5 μM	5 μM	0.16 mg/l
2,4-D	-	1 mg/l	1 mg/l	-
BAP	0.5 mg/l	0.5 mg/l	0.5 mg/l	-
IAA	1mg/l	-	-	1 mg/l
IBA	-	-	-	1 mg/l
NAA	-	-	-	1 mg/l
Phytagel	3 g/l	3 g/l	3 g/l	-
pH	5.8	5.8	5.8	-
Mannitol	-	-	36.43	-
Sorbitol	-	-	40.038	-

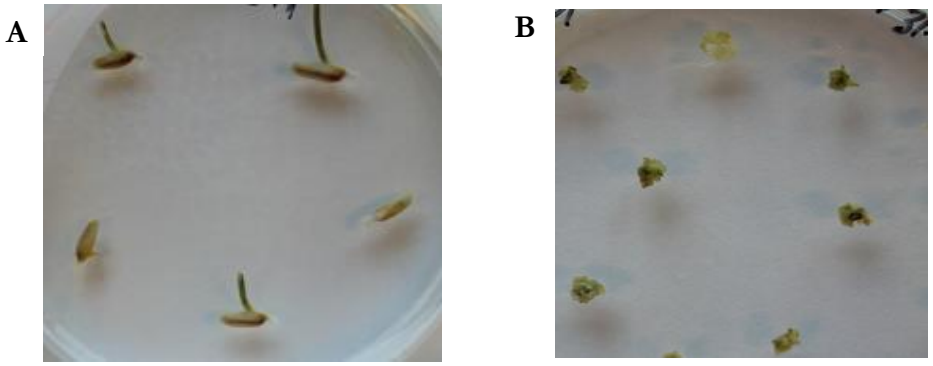


Figure 3.1 Mature oat seeds kept on different MS-based media. (A) Germination roots and shoots are clearly visible, (B) regenerative calli obtained from mature seeds, maintained on DBC3 medium.

3.3.2. *Ac*/*Ds* plasmid constructs

Using the Gene Gun delivery system, different constructs were transformed or co-transformed into oat callus derived from mature oat seeds. These constructs include:

i. *Ds*-Act1-*GUS* (*GUS* Trap) construct (pSP-WDV-Act1-*Ds-Bar-GUS*.nos)(McElroy et al. 1997). This construct consists of a pSP backbone and includes the replication-associated region of wheat dwarf virus (WDV), in addition to a rice actin (Act1) promoter. It also contains the maize *Ds* element borders (254 bp of 5´, and 340 bp of 3´ sequences). A modified *Ds* element interrupts a sequence of the *E. coli* β-glucuronidase coding region (*uidA*), which is followed by the 3´transcriptional termination region (*nos*) of the *Agrobacterium tumefaciens* nopaline synthase gene. The *Ds* portion also contains a coding region of the *Streptomyces hygroscopicus,* phosphinothricin acetyltransferase gene (*Bar*) and (*nos)* terminator sequence (Figure 3.2).

ii. *Ds-Bar* construct: (pSP-*Ds*Ubi*Bar*nos*Ds*) (Koprek et al. 2000). This construct consists of a (*Bar*) gene derived from a maize ubi1 promoter and (*nos*) terminator. The *Bar* gene is flanked by two *Ds* terminals (250 bp in each 3´ and 5´ ends). These sequences were included in a pSP backbone (Figure 3.3).

iii. *Ds-Bar-GUS* activation tagging construct (vec 8)(Ayliffe et al. 2007). This construct contains two maize *Ds* terminus (3´ and 5´) and two full sequences of the maize polyubiquitin promoter with opposite transcription orientations out of both ends. The portion between the two *Ds* termini contains (*Bar*) ORF as a selection marker, under the control of a CaMV35S promoter, and and OCS3 (o) transcriptional termination sequence. Moreover, a *GUS* reporter gene (*uidA*), followed by a *nos* termination sequence is present next to the *Ds* 3´ terminus. Furthermore, a 900 bp fragment of the *Ac* element is included and can be used as a probe binding site to be used in further southern blotting analysis (Figure 3.4).

iv. Ubi-*Ac* construct: (pCambia-Ubi*Ac*nos)(Koprek et al. 2000). This construct consists of a maize Ubi1 promoter directing *Ac*Tpase ORF, and *nos* terminator. These are included in a pCambia backbone (Figure 3.5).

v. GFP-Ubi-*Ac* construct: (pCambia-ActGFP-UbiAcnos)(Singh et al. 2012). This construct is a modification of Ubi-*Ac*, consisting of a transposase gene (*Ac*) driven by the Ubi promoter (pUbi*Ac*), and a GFP gene under an actin promoter control (Act1) expressed in a pCambia 1300 binary vector and fused to a transcription terminal region (*nos*) for both genes. Also, two

selection markers are present for kanamycin and hygromycin antibiotic resistance genes (Figure 3.6).

vi. *hpt* construct **(**pAct1I*hpt*) (Cho et al. 1998a). In this construct, the rice actin1 promoter (Act1), in addition to its intron (Act1I), controls the hygromycin phosphotransferase (*hpt*) gene fused to the transcription terminal region (*nos*) (Figure not shown).

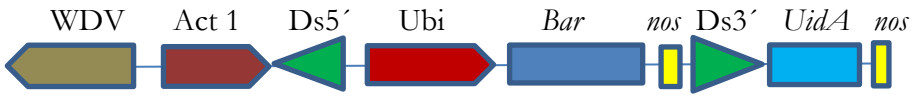


Figure 3.2 Schematic representation of the cassette used in the Act1-*Ds-GUS* construct (pSP-WDV-Act1-*Ds-Bar-GUS*.nos) (*GUS* Trap), indicating the orientation of (Act1), and Ubi promoters. Also shown are the maize *Ds* element borders (254 bp of 5´ and 340 bp of 3´ sequences). A modified *Ds* element interrupts a sequence of an E. coli β-glucuronidase coding region (*uidA*), which is followed with the 3´ transcriptional termination region (*nos*) of the *Agrobacterium tumefaciens* nopaline synthase gene.

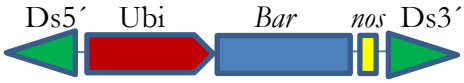


Figure 3.3 Schematic representation of the cassette used in the *Ds*Ubi*Bar* construct (pSP-*Ds*Ubi*Bar*nos*Ds*) including a *Bar* gene deriving by a maize ubi1 promoter and *nos* terminator. The *Bar* gene is flanked by two Ds terminals (250 bp in each 3´ and 5´ ends). These sequences were included in a pSP backbone.

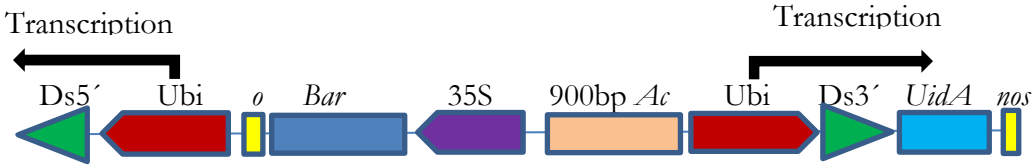


Figure 3.4 Schematic representation of the cassette used in the *Ds*Ubi*Bar*Ubi*DsGUS* construct (*Ds*Bar-*GUS* Activation Tagging Construct) (vec8), indicating the orientation of the Ubi transcription, and the maize *Ac* terminus (5´ & 3´). Also shown is the location of the strong promoter 35S, the two terminations, *i.e.*, *nos* that regulates *GUS* gene, and o that regulates the *Bar* gene, in addition to a 900bp fragment of the *Ac* element, which is included to separate the Ubi inverted repeats.

Figure 3.5 Schematic representation of the cassette used in the pCAMBIA-Ubi*Ac*Tpase construct, consisting of a maize Ubi1 promoter directs (*Ac*Tpase) ORF, and (*nos)* terminator. These are included in pCambia backbone

Figure 3.6 Schematic representation of the cassette used in the pCAMBIA-Ubi*Ac*Tpase-Act*GFP* vector, indicating the orientation of *Ac*Tpase and *GFP* on the construct.

3.3.3. Genetic transformation using particle bombardment

Biolistic equipment (PDS-1000 He, Bio-Rad, Hercules, CA) operating at 900 psi (Lemaux et al. 1996; Cho et al. 1999) was employed. Golden microparticles, with a diameter of 0.6-1.6 µm, and individually 100 ng in weight, were coated with a solution contains one or two of the *Ac/Ds* constructs. Highly regenerative tissues were transferred to osmotic media 4 hours before they were bombarded or co-bombarded. The osmotic media consisted of the same components as DBC3, in addition to 40.038 and 36.43 g L^{-1} of sorbitol and mannitol, respectively (Table 3.1). After 4-24 hours, the bombarded calli were removed to different selection media, based on the selectable markers used in the bombarded constructs. The bombarded callus pieces were maintained in this selection medium at 27 °C in the dark, for two rounds of 2 weeks each. Selected calli pieces were then transferred into non-selective regeneration media, under higher density light (roughly 45–55 µE), at 28 °C and under a 16/8-hour light/dark photoperiod, for 4 weeks. After generating shoots, regenerated calli were transferred again into magenta boxes containing BCI-DM medium (Cho et al. 1999). This medium bore several root inducing hormones (Table 3.1). The regenerated plantlets were maintained in the rooting media until roots were sufficiently developed before being transferred to soil. Thereafter, the plantlets were grown to maturity, under greenhouse conditions, including approximate day and night temperatures of 18°C and 15°C, respectively, under 16 hours of daylight at 1000 µE.

3.3.4. Selection media

Different selection mediums were used based on the selectable marker genes included in the *Ac/Ds* plasmids. In the case of calli bombarded with constructs bearing the *hpt* gene, selection media were supplemented with 30 mg L^{-1} hygromycin B (Sigma), while for those bearing the *Bar* gene, selection media were supplemented with 3 mg L^{-1} of bialaphos (IGNITE®, Bayer Crop Science, Canada).

3.3.5. Biological analyses

3.3.5.1. Histochemical *GUS* assay

Ds-GUS transgenic candidates were tested for β-glucuronidase activity, following the histochemical staining method of Jefferson et al. (1987). Leaf material (4-leaf stage) with its chlorophyll removed in an acetone solution, was then incubated at 37° C overnight in the *GUS* assay buffer. Blue colored

tissues or foci indicated the expression of the *GUS (uidA)* gene and hence the transgenic status of the plants transformed with the *GUS* construct.

3.3.5.2. Basta herbicide resistance test

The *Bar* gene isolated from *Streptomyces hygroscopicus*, encodes a phosphinothricin acetyltransferase protein, which provides the resistance to the Basta (bialaphos) herbicide (Phosphinothricin) (Jones and Sparks 2009; Thompson et al. 1987). Glufosinate ammonium, and IGNITE herbicide (IGNITE®, Bayer Crop Science, Canada), in was applied to the 3-4 leaf stage leaves of oat plants at a concentration of 0.33%. After 5 days of the IGNITE treatment, the painted leaves were explored for symptoms of necrosis, a primary screening for *Ds* plants. Plants with healthy green leaves were selected as positive putative transgenic events and were subjected to additional molecular analysis.

3.3.6. DNA Extraction

As a first step of molecular analysis, samples of young seedlings (3-4 leaf stage) from the T0, T1 generation of oat plants were collected, before being frozen in liquid nitrogen. The CTAB+BME extraction buffer technique was used for DNA extraction. Frozen samples were ground using a Tissue Lyser 2 QIAGEN apparatus, prior to the addition of 500 µl of CTAB+BME buffer. Each sample mixture was incubated in a water bath at 65 C° for 30 minutes, with mixing every 10 mins. After the incubation step, 400 µl of 24:1 Chloroform: Isoamyl alcohol (CIA) were added. The samples were centrifuged down for 6 min at 14000 rpm. From each sample 300 µl of supernatant was removed and placed into a new tube, before 3 µl of RNase solution was added to each supernatant and mixed. These samples were maintained at room temperature for 30 min. Cold isopropanol (400 µl) was added to each sample, then, samples were centrifuged again at 14000 rpm, for 10 min. The supernatant was decanted carefully, without disturbing the DNA pellet. Pellets were washed with 200 µl of cold 70% ethanol, followed by centrifugation for 5 min, at 3300 rpm. A similar washing step with 200 µl of 95% cold ethanol was performed, followed by eluting the DNA pellet in 40 µl of distilled water. The eluted genomic DNA samples were stored at -80° C until future use.

3.3.7. Molecular analysis using Polymerase Chain Reaction (PCR)

Different Polymerase chain reaction (PCR) programs were used to detect the presence of modified *Ac/Ds* elements, the flanking sequence, and the empty donor. This technique was possible due to the known sequences of *Ac/Ds* constructs. Several specific primer sets were used in these programs (Table 3.2). Each PCR reaction contains 1 µl of DNA template, in addition to a mix consisting of 1 µl of each primer, 10 µl of GoTaq® Green Master (Promega Corporation, Canada), and 7 µl of molecular H_2O, forming a total volume of 20 µl per sample. All PCRs were carried out using a program which

followed an initial denaturation at 95°C for 2 min, followed by 36 cycles of 95°C for 30 sec, then an annealing step for 45 sec and an extension step at 72°C for 60 sec. The temperature used in the annealing step varied between 55-60°C, depending on the primers used. An 0.8% agarose gel in 1% TBE buffer served in testing the PCR products by gel electrophoresis.

3.3.8. Growth conditions for putative *Ac/Ds* transgenic lines

The *Ac/Ds* parental plants selected through the tissue culture and transformation process were grown at day/night temperatures of 18°C/15 °C at a light intensity of roughly 1000 µE and under a 16:8 hours photoperiod. Six inches pots having a commensurate mixture of agro and soil were used for growing three to four plants per pot. These were watered daily watering. A standard fertilizer containing a ratio 20:20:20 (N; K; P) was added to the water twice a month.

3.3.9. RNA extraction, cDNA synthesis, and real time PCR

RNA was isolated from green leaves following the method described in Singh et al. (2013). Isolated RNA was treated with DNase, then a 500-ng sample was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad). To exploring for the presence of *Ac*Tpase, PCR was performed using an *Ac*Tpase specific primer set (*Ac*3, *Ac*5). The primers (Table 3.2) were designed from the exonic region of the *Ac* gene which amplified the 825 bp product.

3.3.10. Optimization of TaqMan qPCR for copy number variation (CNV) in transgenic oat

3.3.10.1. Selection of a stable reference gene for the TaqMan CNV assay in oat

Copy number was estimated for the transgenic events-containing the *Bar*-selectable marker, using a qPCR TaqMan assay (Ingham et al. 2001), but using a single-plex assay rather than a multiplex one. The *Acc1* (*Avena sativa* acetyl-CoA carboxylase) gene served as a reference (Kianian et al., 1999). This gene is stable and has a single copy in each chromosomal set (Yan et al., 2014). The (*TNP-28*0) mutant, a barley line carrying a single copy of the *Ds* insertion was chosen as a calibrator.

3.3.10.2. Primers and Probes

Primers and probes, of both the reference gene (*Avena sativa Acc1*), and targeted gene (*Bar*), were designed manually, following the Integrated DNA Technologies (IDT), (Coralville, IA, USA), qPCR application guide. Both probes were labeled at their 5' end, with HEX fluorophore as a reporter dye, and Iowa black at the 3' end, as a quencher dye. Sequences of TaqMan based probes and primers are shown in Table 3.2.

3.3.10.3. TaqMan qPCR reaction

TaqMan qPCR reaction was performed following the method of Ingham et al. (2001). A total of 26.7 µL of gDNA (10 ng μL^{-1}), was mixed with 30 µl of 2 × TaqMan Universal PCR Master Mix (Applied Biosystems), along with a final concentration of 0.9 nM of each primer, and 0.1 nM of each probe, in a total volume of 70 µl. For each sample, two reactions were prepared. The first reaction included the primers and probe specific for the target *Bar* gene, while the second one was specific for the endogenous reference gene (*acc1*). Each reaction was divided into three replications of 20 µl. Cycling conditions of TaqMan qPCR, implemented on a Stratagene Mx3005 instrument, included three steps; a first cycle at 50°C for 2 minutes, a second cycle at 95°C for 10 minutes, followed by 35 cycles of 15 sec at 95°C and 1 minute at 60 °C.

3.3.10.4. *Ac/Ds* copy number calculation and estimation

The copy number (CN) of the *Bar* target gene in each oat sample, was calculated based on the Ct value obtained through the qPCR for both *Bar,* and *acc1* genes (Ingham et al. 2011). In our assay, for each sample, if the variation in Ct variation exceeded 0.5 across the triplicate reactions, it was excluded and repeated. The Ct value of oat *Ds* transformants, and of the barley (calibrator) line were first normalized by comparing them to the Ct value of the endogenous reference gene (*acc1*), using the formula ΔCt = Ct of target gene (*Bar*) - Ct of the endogenous reference gene (*acc1*)). After calculating the difference in ΔCt as ΔΔCt = (ΔCt unknown – ΔCt *acc1* known), the copy number for each sample was calculated as (Copy number of *Bar* = CNc = $2^{-\Delta\Delta Ct}$). The *Bar* gene copy number is an indicator of the CN of the modified *Ds* element, since the *Bar* gene is present between the two *Ds* terminal inverted repeats (TIRs). Our estimation of CN (CNe) followed the criteria: (*i*) if CNe ≤ 1, then 1 copy, (*ii*) if 1< CNe ≤ 2, then 2 copies, if CNe 2 < CNe ≤ 3, then 3 copies, and so on.

3.3.11. Generation of populations for transposon activation

3.3.11.1. Self-pollination of transgenic lines containing both *Ac* and *Ds* elements

Transgenic lines containing both *Ac* and *Ds* elements were generation advanced through self-pollination. Mature seeds were collected and then planted in varied numbers, based on the quantity of seed obtained from each plant. T1 plants were grown under greenhouse growth conditions (see section 3.3.11). At the 3-4 leaf stage, genomic DNA was extracted from green leaf tissues as described above, and transposition events were evaluated using different primers (Table 3.2) in the PCR analysis as described in section 3.3.10.

3.3.11.2. Re-activation of *Ds* transposons through the transient activity of Ac-transposase by the biolistic method

The transgenic line (OT22) bearing an intact activation tagging *Ds-GUS* (Vec 8) construct, served as a donor of *Ds* explants. Mature seeds were used for generation of calli. After three weeks, well regenerated green callus pieces were bombarded with the Ubi*Ac*Tpase-*GFP* construct using the biolistic system as (see section 3.3.6). After 2-3 days, the expression of the *GFP* was detected using fluorescence microscopy. In this system, a Nikon SMZ1500 stereomicroscope supported by a *GFP*-1 filter was used, using an excitation wavelength of 480±40 nm. After detecting the expression of *GFP* (Figure 3.18), the positive bombarded calli were sub-cultured and putative transgenic plants were regenerated. Similar to the previous population, genomic DNA was extracted from their green leaf tissue, then screened for transposon activity through various PCRs.

3.3.12. Molecular screening for transposition activity

Different primer sets (Table 3.2) were used to detect the presence of *Ds*, *Ac*, and *Ds* inside its original site (flanking sequence PCR), in addition to the *Ds* launch site (Empty Donor PCR) (Table 3.2). Stable transposition events were selected based on the following criteria: (*i*) the presence of the *Ds* outside its original site, and (*ii*) absence of the *Ac* element. The segregation of the *Ds* element far from the *Ac* element provided stability to the *Ds* in its alternative site. Thus, such *Ds* transposition events could be selected as stable mutants.

In the case of the empty donor analysis, the primers used were designed from the backbone sequences close to the *Ds5'*, and *Ds3'* termini. This test provided evidence of the transposition event having occurred, due to the absence of the *Ds* element from its original site.

Table 3.2: Primers and probes used in PCR-based analysis, including the detection of *Ds* sequence, *Ac*Tapse sequence, *Ds* flanking sequence, the empty donor (*Ds* launch site), and in addition of the primers and probes used in Inverse, HE-TAIL, Adapter Ligation, and Q PCRs

Primers	**O**	**Sequence (5'→ 3')**	**Details**	**Size (bp)**
MDS5F	F	AGGGATGAAAACGGTCGGTA	Native *Ds*	125
MDS5R	R	ACCGTATTTATCCCGTTCGTT		
HPTF	F	AAGCCTGAACTCACCGCGACG	*Ds GUS* (vec8) & *hpt* in Backbone	725
HPTR	R	AAGACCAATGCGGAGCATATAC		
JNOSF	F	GCGCGGTGTCATCTATGTTACTAGAT	*Ds* (*Ds*Ubi*Bar*)	248
JDS3R	R	TATCCCGATCGATTTCGAAC		
Qnosf	F	AATCCTGTTGCCGGTCTT	*Ds* (*Ds*Ubi*Bar*)	473
QDS3R	R	CGTACCGACCGTTATCGTAT		
Ds5F	F	GATCCGGTCGGGTTAAAGTC	*Ds* (*Ds*Ubi*Bar*)	813
UbiR	R	CAGGCTGGCATTATCTACTCG		
AC5'	F	AACCTATTTGATGTTGAGGGATGC	*Ac*Tpase &	825

AC3'	R	ACCACCAGCACTGAACGCAGACTC	*Ds* (vec8)	
ubiDs-uidA F2	F	GGTTGGGCGGTCGTTCATTC	*Ds* (vec8)	1113
ubiDs-uidA R2	R	GCGGGATAGTCTGCCAGTTC	Flanking	
EDS5	F	CGTCAGGGCGCGTCAGCGGGTGTT	*Ds* (*Ds*Ubi*Bar*)	297
JIPR5	R	TTCGTTTCCGTCCCGCAAGT	Flanking	
JIPF1	F	AACTAGCTCTACC GTTTCCG	*Ds* (*Ds*Ubi*Bar*)	444
JEDS3	R	TGCTCACATGTTCTTTCCTGCG	Flanking	
EDS5	F	CGTCAGGGCGCGTCAGCGGGTGTT	*Ds* (DsUbi*Bar*)	490
EDS3	R	TGCTCACATGTTCTTTCCTGCG	Empty Donor	
Vec8-5-F2	F	GATAGCTGGGCAATGGAATCC	*Ds* (vec8)	671
UbiDs-UidAR2	R	GCGGGATAGTCTGCCAGTTC	Empty Donor	
QPCR_BarF	F	GAAGGCACGCAACGCCTAC	TaqMan QPCR	119
QPCR_BarR	R	CTCCAGGGACTTCAGCAGGTG	TaqMan QPCR	
QPCR-acc-F2	F	CACGGCTGATCTATGTGCTGC	TaqMan QPCR	138
QPCR-acc-R2	R	TGCCGAAGAATCGGCTCTTCC	TaqMan QPCR	
QPCRBarprobeF	F	ACGGGACTGGGCTCCACGCTTCTA	TaqMan QPCR	
QPCRaccprobe2	F	TGGAGCACTCATGCCTATGCGCCG	TaqMan QPCR	
JIPF4	F	CTCGTGTTGTTCTGAGCGCACACA		
JIPR3	R	CGACCGGATCGTATCGGT		
JIPF2	F	CATATTGCAGTCATC CCGAA		
JIPR9	R	TGCGGAACGGCTAGAGCCAT	IPCR	
JIPF7	F	CTAGATCGGCGTTCCGGT		
JIPR5	R	TGCGGAACGGCTAG AGCCAT		
JIPF1	F	AACTAGCTCTACC GTTTCCG		
JIPR6	R	TAGCAGCACGGATCTAACAC		
AC946R		CAATTACAGGCAAGCCATCC		
Ds 379R		TGCACTGCAGGAATTCGATA		
Ds 3253F		TGCAGTCATCCCGAATTAGA		
Ds 119R		TTATCCCGTTCGTTTTCGTT	AL PCR	
Ds 3325F		ACGGTCGGGAAACTAGCTCT		
Ds 80R		GTTTCCGTCCCGCAAGTTA		
Ds 3435F		CGAAATCGAGGATAAAAC		
QTLAD1-1		TAGCGGCTGAAGCACCTGCAGGCVNVN NNGGAA		
JNOSF		GCGCGGTGTCATCTATGTTACTAGATC		
QTLAD1-2		TAGCGGCTGAAGCACCTGCAGGCBNBN NNGGTT		
QDS1-5		CCCGTCCGATTTCGACTTTAACCC		
QTAC1		TAGCGGCTGAAGCAC		
QTPF2		TAGCGGCTGAAGCTGCCTGCAGGAAAC GGTCGGGAAACTAGCTC	HE-TAIL PCR	
QTPR1		TAGCGGCTGAAGCTGCCTGCTCGACCG GATCGTATCGGTTTTCG		
JDSB3		TGTATATCCCGTTTCCGTTCCGTT		
JIPR5		TTCGTTTCCGTCCCGCAAGT		
JIPF8		TATACGATAACGGTCGGTACGG		
QDS4-5		CGACCGTTACCGACCGTTTT		

3.3.13. Generation of *Ds* flanking sequences

Isolation of the flanking sequences adjacent to *Ds* insertion sites was performed using different techniques, including Nested Inverse PCR (Singh et al., 2012), HE- Tail PCR (Tan and Singh 2011) and Adapter Ligation PCR (ALPCR) (Brown et al. 2012).

For the Nested Inverse PCR (IPCR) method, an Inverse PCR method (Ochman et al. 1993), with slight alterations by Cooper et al. (2004) and Singh et al. (2012), was used. Suitable enzymes were used to digest the genomic DNA in highly diluted form. After digestion, the restriction enzymes were subjected to heat deactivation at 65°C. Digested DNAs were purified using a phenol/chloroform (1:1) protocol followed by self-ligation using T-DNA ligase (New England Biolabs, CA). The restricted ligated product was used for a series of nested PCRs. The first PCR reaction contained approximately 0.1 µg of the ligated purified DNA, 0.25 µl of 1 unit of ExTaq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 2.5 µl of a 2.5 mM deoxynucleotide triphosphates (dNTP) mixture, and 2.5 µl of 10 × buffer, in addition to 0.5 µl (10 µM) of the first set of nested primers JIPR3 and JIPF4 for the 5' terminus, and JIPR9 and JIPF2 for the 3' terminus (Table 3.2). PCR1 products were purified and diluted to 50× for the 3' terminus, and to 20× for the 5' terminus, and each used as a template for the second stage of PCR. For the second PCR, nested primers JIPF7 and JIPR5 for the 5' side, and JIPF1 and JIPR6 for 3' side (Table 3.2), were used rather than the previous PCR1 primers, but in a similar reaction. Gel electrophoresis on a 1% agarose gel in 1% TBE buffer served to test the PCR products. Bands observed on the gel were gel-purified, utilizing a gel extraction kit (QIAquick® Gel extraction kit, Qiagen, Valencia, CA, USA).

In the HE-TAIL PCR (Tan and Singh 2011) method, four separate PCR runs were applied, using 10× PCR buffer containing 2.5 mM deoxynucleotide (dNTPs) solution mix, and 10 mM $MgCl_2$, in addition to genomic DNA, and Takara ExTaq DNA polymerase (Takara-Bio, Dalian, China). A series of PCRs was performed and included pre-amplification, primary, secondary and tertiary amplification using a GeneAmp® PCR System 9700 system, as described by Tan and Singh (2011).

In the adapter ligation method (ALPCR), the genomic DNA was separately cut by four different restriction enzymes (HinfI, AseI, HhaI, AvaII). These enzymes were chosen based on the restriction position in the (*Ds*Ubi-*Bar*) sequence, with no further restriction site being present in our cassette sequence. Fragmented genomic DNA was then ligated to specific adapters that fit the restriction sites. These adapters are designed to contain appropriate overhangs, and a specific primer binding site (AC946R). After ligation, three consecutive rounds of nested PCR with three different primer sets

were carried out (Table 3.2). A similar Adapter-Ligation technique, using a Universal Genome Walker 2.0 kit (Takara Bio, USA) was used for isolating *Ds* insertion flanking sequences.

3.3.14. Bioinformatic analyses

Bioinformatic analyses of isolated flanking sequences were carried out using a basic local alignment search tool (BLAST) algorithm, and compared with references in the HarvEST database http://harvest.ucr.edu, Gramene http://www.gramene.org/, the oat genome Project http://www.avenagenome.org/, and NCBI http://www.ncbi.nlm.nih.gov/.

3.4. Results

3.4.1. Does the oat genome contain endogenous *Ac*/*Ds* type transposons?

As both maize *Ac* and *Ds* transposons are well characterized, molecular screening using PCR was applied on the cultivated oat 'Park' genotype, in which two sets of primers (Ac3´ and Ac5´), and (MDs5R, MDs5F) were used to amplify conserved sequences from both Maize *Ac*, and *Ds* transposons. No amplification products were observed, confirming the absence of *Ac* and *Ds* type elements in the cultivated oat genotype (Figure 3.7). Moreover, we used basic bioinformatic tools (blasting) to detect the presence of homologous sequences to the two inverted repeats *Ds3, Ds5*, in addition to the *Ac*Tpase coding sequence. No significant homologous sequences were observed in the oat Genome Project Database. Since *Ac*/*Ds* elements were found to be missing in the oat genome, this result enabled us to introduce the heterologous maize *Ac*/*Ds* system into the oat genome.

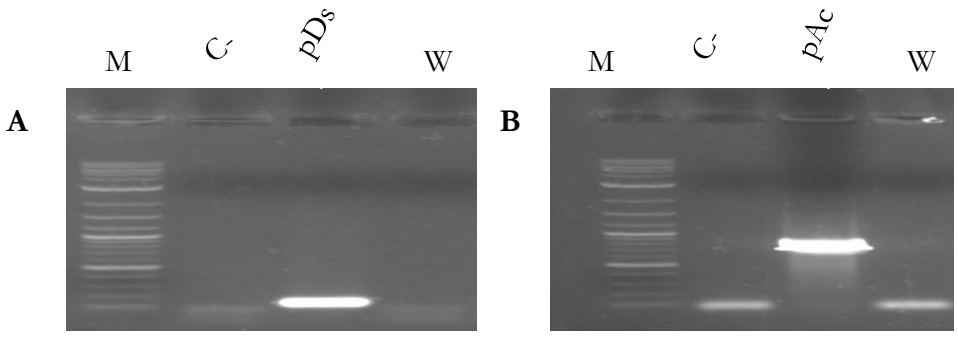


Figure 3.7 PCR analyses to identify the presence of homologous *Ac*, and *Ds* maize elements in the common oat genome. In both *Ds*Ubi*Bar (*pDs) and UbiAcTpase (pAc) specific PCRs, four samples were used, including 1) non-transgenic oat (C-), either p*DS* or p*Ac* specific plasmids, in addition to the water (W). (M) is 1kb plus DNA marker (Invitrogen, Thermo Fisher Scientific, USA). The absence of the specific amplicons in the non-transgenic oat indicates the absence of homologous maize *Ac* and *Ds* elements in the oat genome.

3.4.2. Transient activity assay of maize *Ac/Ds* transposon system in oat

The activity of the *Ac* / *Ds* system was confirmed by co-transformation of Ubi-*Ac* and *Ds* interrupted *GUS* (Act1-*Ds*-*GUS*) (*GUS* Trap) constructs. The presence of *Ds* element directly after the Actin1 blocks GUS gene expression. Ubi-*Ac* was used as a source of *Ac* transposase protein, capable of catalyzing the excision of the *Ds* element from its location. The *Ds* transposition can initiate the expression of the *GUS* gene, and therefore produce the β-glucuronidase enzyme. Oat (cv 'Park') mature seeds were used as explants for regeneration of embryogenic calli. A histochemical β-glucurodinase (*GUS*) assay was conducted on calli pieces 48 hrs after the co-bombarded experiment. Of calli pieces bombarded with both *Ac* and *Ds*-*GUS* trap constructs, 85% showed large numbers of blue foci indicative of positive *GUS* expression; however, no blue foci were observed in either untransformed calli or calli only bombarded with the *GUS* construct (Figure 3.8). Blue foci were only expected if *Ds* had moved from its plasmid cassette, thereby enabling the *GUS* gene to be expressed. This transposition activity confirmed that the *Ac* / *Ds* system had the potential to work in the oat genome.

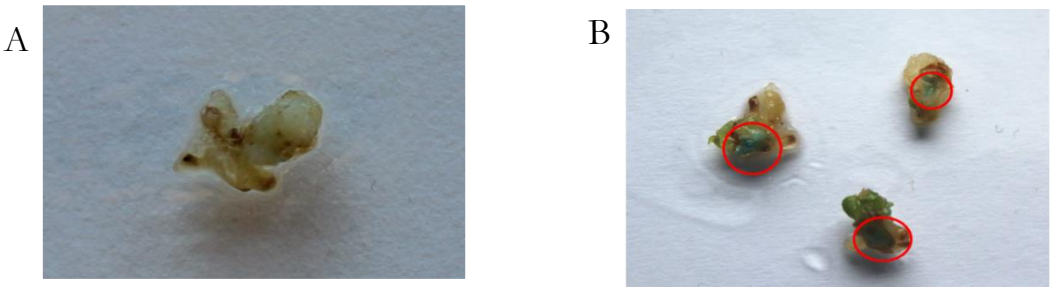


Figure 3.8 *GUS* assay: (A) non-transformed embryogenic callus tissue, (B) represents transformed calli, where red circled portions represent *GUS* gene expression as blue color spots.

3.4.3. Successful transformation of oat with gene constructs containing *Ac/Ds* elements

A total of 2035 calli pieces were bombarded or co-bombarded using several *Ac/Ds* constructs (Table 3.3), prior to their placement on selection medium (Figure 3.9). In this selection process, either bialaphos or hygromycin was used, based on the selectable marker presented in the *Ac/Ds* constructs used in the transformation experiment. In the case of bialaphos, up to 24.8% of the bombarded calli passed the selection and maintained their regeneration ability, while only a maximum of 6.66% passed the hygromycin-based selection process. The selected OCPs were transferred into rooting medium and shoots and roots were observed within 4-8 weeks (Figure3.10). These plantlets were then transferred into soil and grown under controlled conditions in the growth chamber first, then transferred to the greenhouse.

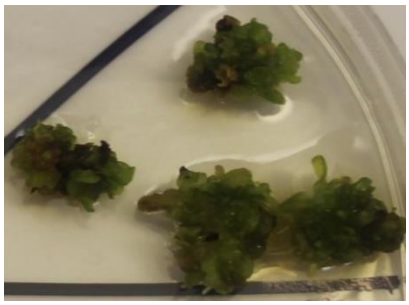
A  B 

Figure 3.9 Bombarded calli after two rounds of selection (four weeks). (A) Represents the putative transgenic calli that passed the selection medium, based on the selection marker used, while the remaining non-transgenic calli died (B).

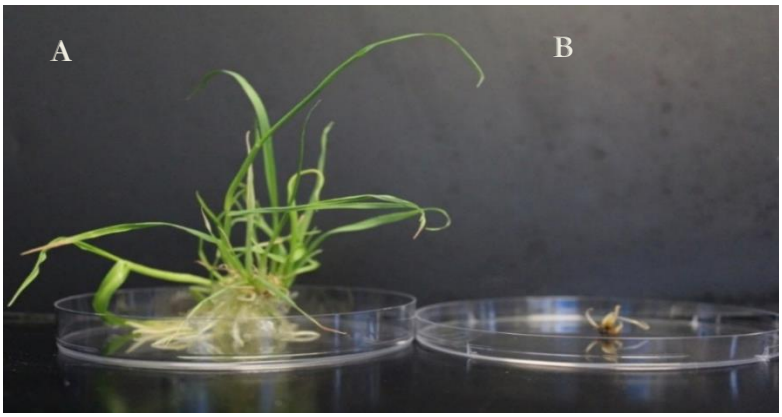


Figure 3.10 An example of putative regenerated transgenic plantlet. (A) After two rounds of selection medium, followed with rooting medium for 4 weeks, while (B) represents an explant that passed the selection medium, but was not able to develop roots, and thus died.

3.4.4. Biochemical assays to confirm the transgenic status of oat transformants

Putative *Ac/Ds* transgenic plantlets, that passed the selection, were subjected to a *GUS* assay in case of a vec8 construct containing the *uidA* gene, and a Basta herbicide painting test in case of all constructs containing the *Bar* gene. In the case of the *GUS* assay, the *uidA* gene expression indicator, blue colored spots or strips in the leaves tested were monitored in two lines, while the negative control showed a completely white color. These lines were selected as putative *Ds-GUS* (activation tagging construct) transgenic lines (Figure 3.11L). In the case of Basta painting test, the presence of the *Bar* gene in the plant provided resistance to the herbicide. The negative control showed necrotic symptoms on the painted portions of leaves. The painted leaves of twenty plants remained green and healthy (Figure 3.11R), indicating the presence of *Bar* gene-related constructs in these plants. Accordingly, these were selected for further analyses.

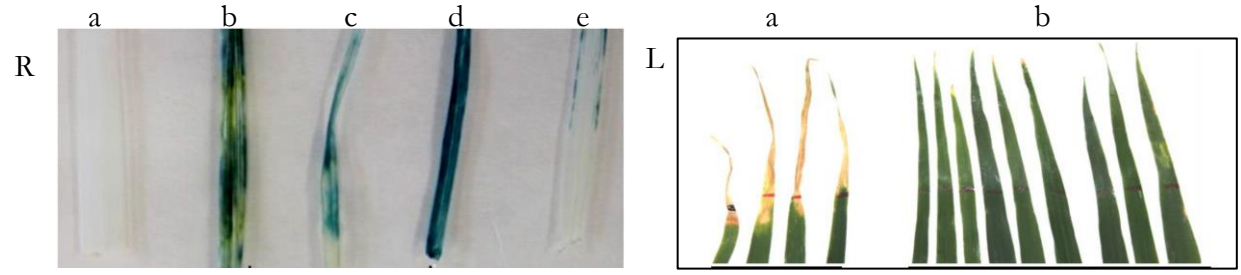


Figure 3.11 Biological analyses of putative transgenic events, **(R)** represents *GUS* histochemical staining of leaf tissues derived from T0 *DsBar-GUS* (vec8) transgenic oat plants (b, c, d, e). Both b and c were derived from line 1 and both d and e were derived from line 2. (a) Represents leaf tissues derived from a non-transgenic oat plant (negative control). The blue color of the transgenic plants' leaves, after applying the *GUS* Assay, confirm the presence of the *GUS* Gene, and therefore Vector 8, confirming these as successful transgenic plants. **(L)** Represents the results of the Basta painting test and confirms those transgenic plants bearing the *Ds*Ubi*Bar* construct. The *Bar* Gene provides resistance to Basta (balaphos) herbicide (Phosphinothricin). This resistance keeps the leaves healthy after painting with the herbicide (b). Comparatively, non-transgenic plants' leaves died (a).

3.4.5. Molecular screening of putative *Ac*/*Ds* transgenic lines

Polymerase Chain Reaction (PCR) sets using different primers sets (Table 3.2) were carried out for further confirmation of the plants' transgenic status. The expected amplicon size was detected in all 24 putative transgenic plants. Among these transgenic events, two events were positive for *Ds-GUS* (vec8), two for Ubi-*Ac*Tpase, thirteen for *Ds*Ubi*Bar*, and seven for Ubi-*Ac*Tpase and *Ds*Ubi*Bar* constructs (Figure 3.12, 3.13, 3.14).

The molecular screening results confirmed the previous histochemical observations in the same plants. As a result, transformation frequencies of up to 19% and 1.9% were achieved using bialaphos, and hygromycin selection media, respectively (Table 3.4).

Unfortunately, no seeds were obtained from either *Ac* lines. Of the 22 remaining transgenic lines all were self-pollinated, and seeds collected. At least five seeds per plant were planted under greenhouse conditions, and similar histochemical and molecular analyses were applied to confirm the integration of our exogenous transposons into the oat genome. Our analysis of T1 plants showed similar results for both the histochemical analysis, and the expected PCR band sizes. For example, screening 31 seedlings derived from two *Ds-GUS* lines, showed blue foci in 14 of them indicating a 58% integration frequency of *Ds-Gus* in the oat genome (Figure 3.16). The presence of *Ds-Gus* in these plants was confirmed using PCR (Figure 3.15).

Table 3.3 The transformation status of heterologous *Ac*/*Ds* in common oat (*Avena sativa*), including the constructs bombarded, selectable and reporter genes used, the number of the plates and calli pieces shot, number of calli passing the selection, and regenerated, as well as the selection media used, and results of biological and molecular analyses.

Construct	Selection gene	Plates	Calli Pieces	Calli Passed Selection	Plantlets Regenerated	Transformants (events)	
						Basta+ PCR+	Gus+ PCR+
Ds-GUS	*hpt* +*GUS*	9	315	6	6	NA	2
Ubi-*Ac*Tpase +*GFP* +*Ds*Ubi-*Bar*	*Bar*	3	105	14	7	0	0
Ubi-*Ac*Tpase +*Ds*Ubi-*Bar*	*Bar*	6	210	41	32	22	NA
Ubi-*Ac*Tpase +pActint*hpt*	*hpt*	12	425	19	14	NA 2	NA
*Ds*Ubi-*Bar* +pActint*hpt*	*hpt*	9	315	2	2	NA 0	NA
Ubi-*Ac*Tpase-*GFP* +*Ds*Ubi-*Bar*	*hpt*	8	280	13	5	0	NA
Ds-GUS + Ubi-*Ac*Tpase +*GFP*	*PMI*	3	105	4	4	NA	0
+ Ubi-*Ac*Tpase +*GFP*	*PMI*	8	280	31	12	NA 0	NA 0
Ubi-*Ac*Tpase *Ds-GUS*	*Bar*	16	560	22	4	0	NA

Table 3.4: Transformation frequency based on the selection medium used, including the constructs bombarded, the number of the calli pieces shot, selection media used, in addition to the transformation frequency.

Constructs	**Calli pieces**	**Selection Medium**	**Transformants OTs**	**Transformation Frequency**
Ds-GUS	175	Hygromycin	2	1.14 %
*Ds*Ubi-*Bar*+Ubi*Ac*nos	210	Bialaphos	20	9.52 %
Ubi-*Ac*Tpase+pActint*hpt*	105	Hygromycin	2	1.9 %

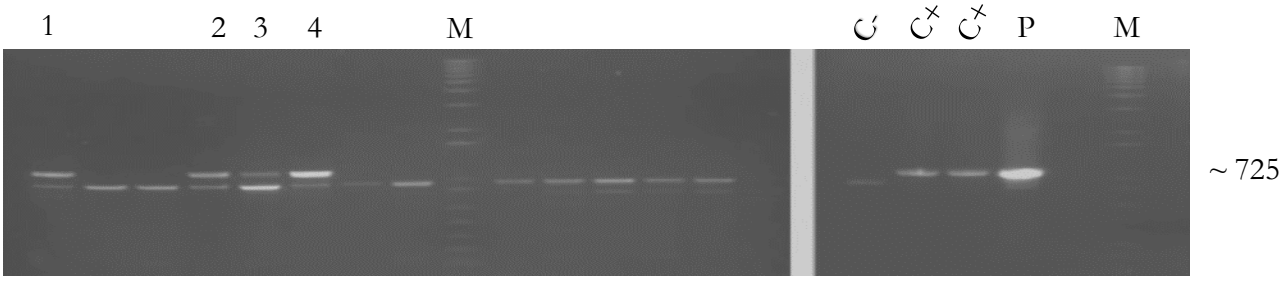


Figure 3.12 PCR analysis of transformed plants using hygromycin (*hpt*) specific primers (HPTF, HPTR). Plants in lanes 1, 2, 3 and 4 are confirmed transgenic plants, which contain larger sized expected bands. The lane C- represents non-transgenic oat, C+ is positive control, and (P) is the *Ds*Bar*GUS* (vec8) construct added as another positive control. (M) is a 1 kB plus DNA marker.

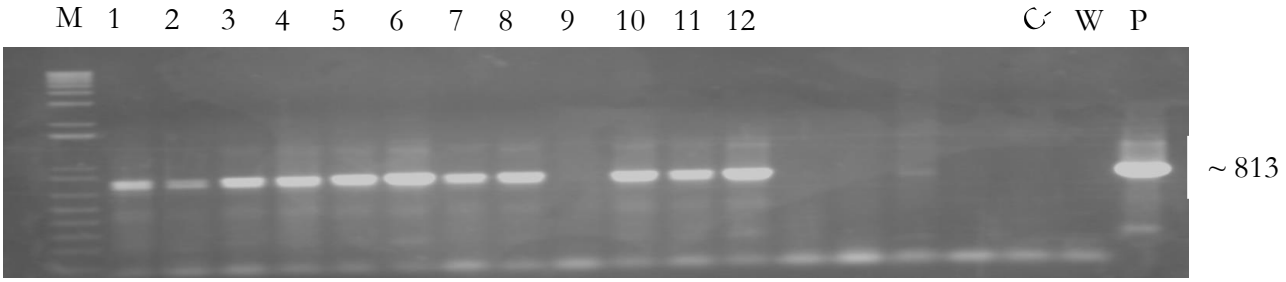


Figure 3.13 PCR analysis of putative transgenic plants co-transformed with *Ds*Ubi*Bar* and Ubi-*Ac*Tpase using *Ds* transposon-specific primers (Ds5F, UbiR). Plants in lanes (1-8), and (10-12) are transgenic and contain the *Ds* transposon. The *Ds*Ubi*Bar* (P) plasmid was used as a positive control, while a non-transgenic oat line (C-), and water (W) were used as negative controls, and (M) was a 1kb plus DNA marker.

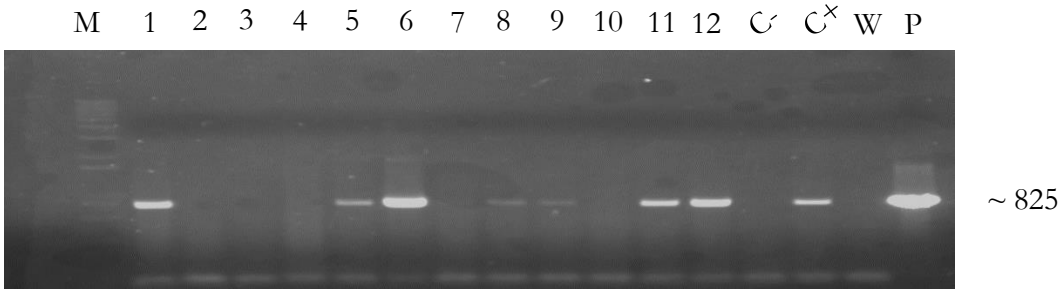


Figure 3.14 PCR analysis of putative transgenic plants co-transformed with *Ds*Ubi-*Bar* and Ubi-*Ac*Tpase using *Ac* transposon-specific primers (Ac3′, Ac5′). Plants in lanes (1, 5, 6, 8, 9, 11,12) are transgenics and bear the *Ac* transposon. The Ubi-*Ac*Tpase plasmid (P) was used as positive control, while a non-transgenic oat line (C-), and water (W) were used as negative controls, and (M) was 1kb plus DNA marker

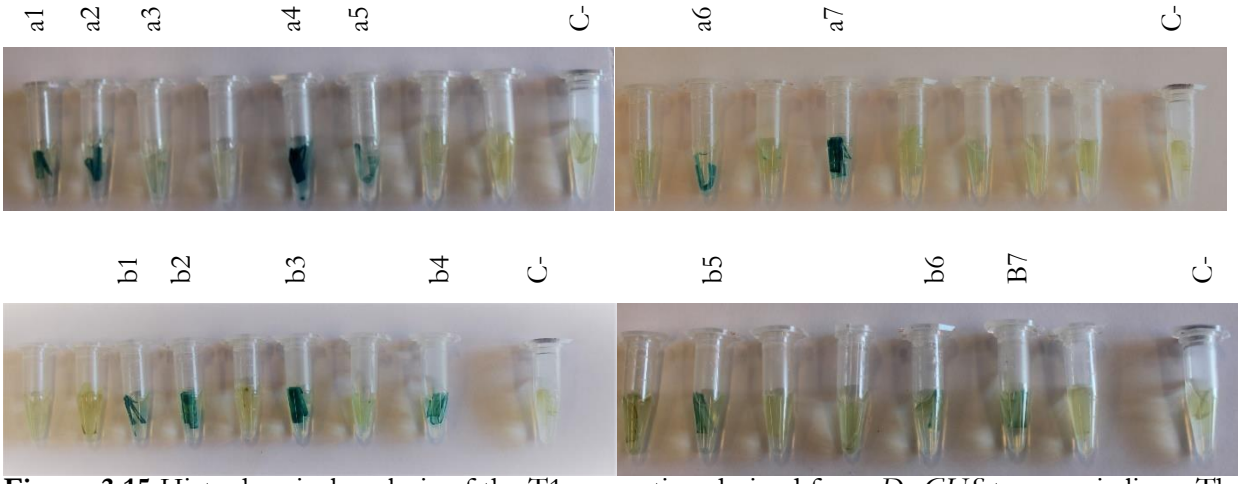


Figure 3.15 Histochemical analysis of the T1 generation derived from *Ds-GUS* transgenic lines. The blue color in the samples (A1-7), and (B1-7) indicates to the presences of the *GUS* gene, and hence the integration of the *Ds-GUS* cassette in the genome of T1 oat lines.

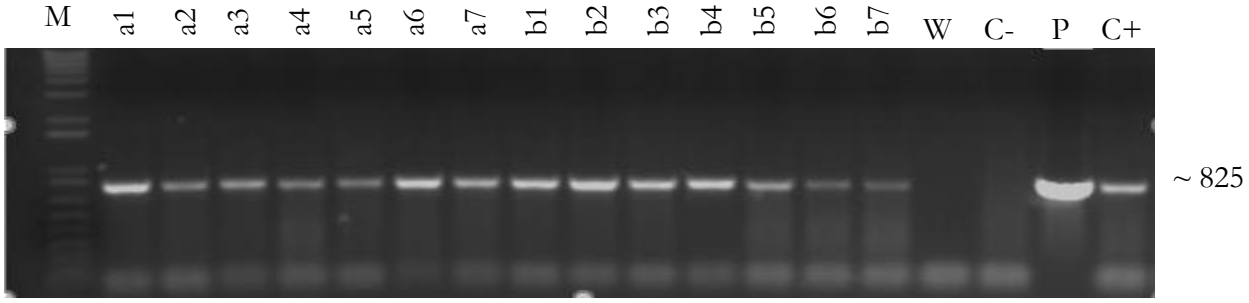


Figure 3.16 PCR analysis of selected T1 generation-lines, derived from T0 *Ds-GUS* transgenic events, using specific primers (Ac3́, Ac5́). The specific amplicon (~ 825 bp) indicates the presence of the *Ds-GUS* construct in lines A1-7 and B1-7. The *Ds-GUS* construct is used as a positive control, while non-transgenic oat is used as a negative control in addition to the water. (M) is 1kb plus DNA marker. These samples correspond to Figure 3.15.

3.4.6. Transposition events and their efficiency in different *Ac/Ds* populations at different generations

3.4.6.1. Reactivation efficiency by generation advance of lines containing both *Ac* and *Ds* elements

Populations from *Ac* and *Ds* element-bearing plants were developed as follows: six transgenic lines containing both heterologous Ubi-*Ac*Tpase and *Ds*Ubi-*Bar* elements, *i.e.,* in the same line, were self-pollinated and seeds collected. These seeds were planted again in various numbers, based on the number of seeds obtained from each *Ac/Ds* line, under greenhouse conditions (Figure 3.17). After 3-4 weeks, green leaf tissues were separately collected from the young seedlings, and genome DNA isolated. A total of 278 samples was screened for possible *Ds* transposition events.

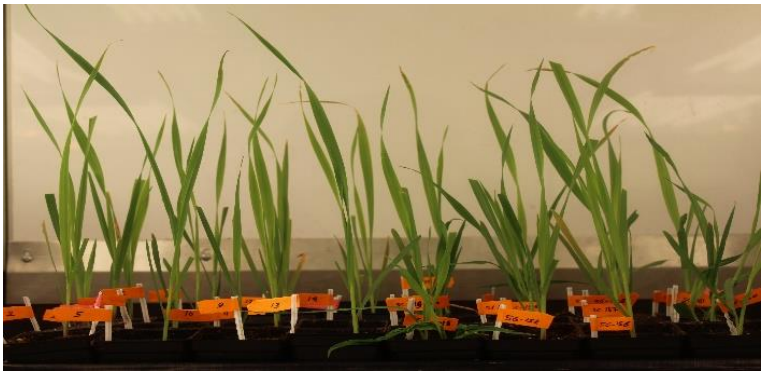


Figure 3.17 Population development of *Ds* remobilization screening by direct planting of mature seeds in the soil. These seeds were generated by self-pollination of lines contain both *Ac*Tpase, and *Ds-Bar* elements.

3.4.6.2. Success in vitro remobilization of Ds with transient Ac Transposase activity

Calli were induced from mature seeds of *Ds-GUS* transgenic line (TO23), which were bombarded with the Ubi*Ac*Tpase-GFP construct in order to provide *Ac* transposase. The objective behind this experiment was the exploration of the transposition activity of the *Ds* element under the transient expression of *Ac*Tpase. As recommended in previous studies (Singh et al., 2012; Sparkes et al., 2006), the transient expression of the GFP gene in bombarded OCPs allowed us to identify calli with AcTpase expression, as the AcTpase and GFP genes have been fused together. This activity was carried out 2-3 days after the bombardment process. Bright green bright spots were monitored in the bombarded calli exposed to UV radiation (Figure 3.18). The bombarded calli were transferred directly to regeneration medium for two weeks, followed by rooting medium, before transferring the plantlets into soil, under greenhouse conditions (Figure 3.19). A total of 63 plantlets was regenerated and screened for putative *Ds* transposition activity.

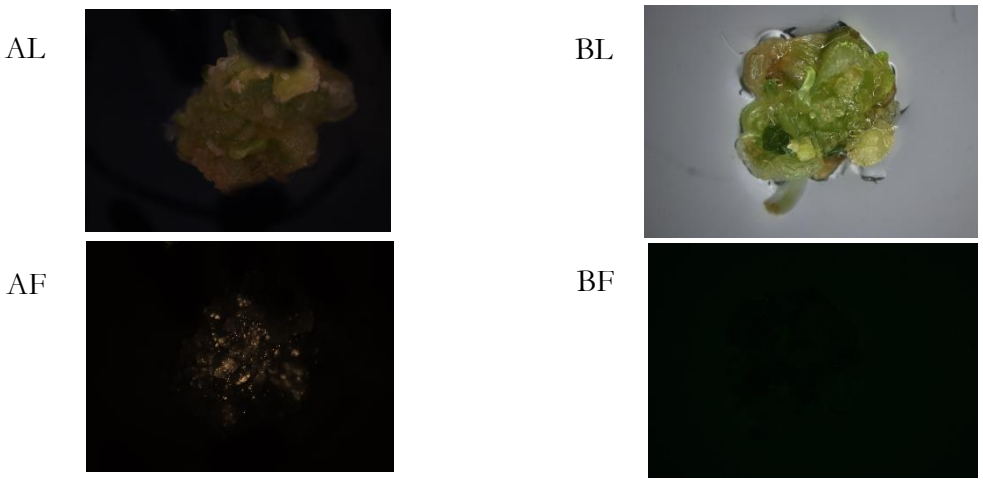


Figure 3.18 GFP expression in bombarded calli under fluorescence microscopy. (AL) and (BL) represent the putative transgenic and the non-transgenic calli, respectively under regular white light. (AF) and (BF) represent the same two calli exposed to fluorescence inducing light. Bright green foci in the transgenic calli (AF) indicates GFP expression, whereas the absence of such symptoms in the non-transgenic calli (BF) indicates the lack of GFP expression.

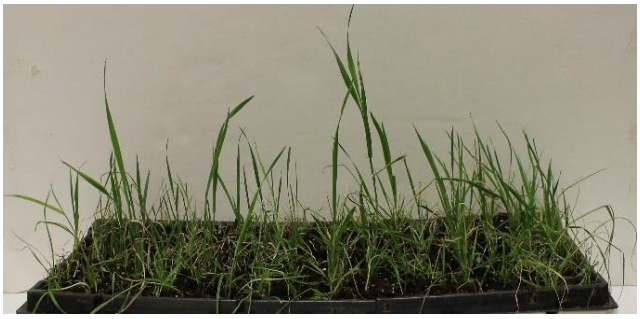


Figure 3.19 Population development of *Ds* remobilization screening by regeneration of calli derived from parental *Ds-GUS* (vec8) transgenic lines. These calli pieces were bombarded with the Ubi*Ac*Tpase-GFP construct before they were *in vitro* regenerated into plantlets.

3.4.7. Observation of the Ds transposition activity in the oat genome

Ds is a non-autonomous element, and *Ac*Tpase expression is essential to *Ds* movement (Fedoroff et al. 1983). Thus, firstly, two T0 *Ac*/*Ds* transformant lines were randomly selected to detect the *Ac*Tpase transcript abundance in these lines. Figure 13.20 shows the amplicon size detected in cDNA derived from these two lines, which reflects the *Ac*Tpase expression required for the activation of *Ds* remobilization in oat transformants.

Molecular analysis of *Ds* element mobilization was carried out using three categories of experiments: (*i*) *Ds,* and *Ac* PCRs confirming the presence of either both elements or one of them, (*ii*) flanking *Ds* PCRs detecting *Ds* transposition outside its original site, and (*iii*) empty donor PCRs exploring the

launch excision site resulting from *Ds* movement. The stable *Ds* transposition event was selected if the screening of the *Ac*, *Ds*, and flanking *Ds* showed negative, positive, and negative results, respectively. For example, both OTs lines showed bands in *Ds*-related PCR, and an absence of bands in both *Ac*, and flanking *Ds* PCRs (Figure 3.21). These results represent the *Ds* element being transposed from its original construct, into an alternative genomic site, and segregated far from the *Ac*Tpase element in both OTs.

The amplicon size of the PCR product varied based on the different primers used (Table 3.2). Firstly, the parental transgenic lines generated were subjected to the same PCRs series for primary transposition identification. A primary transposition frequency of 5% was observed at the tissue culture stage (somatic transposition). This somatic transposition event likely occurred due to the extrachromosomal expression of *Ac* transposase, under tissue culture conditions. Moreover, a total of 341 plants, generated from parental transposon transgenic lines, were screened using the previous PCRs categories. The transient activity of *Ac*Tpase generated up to a 15.9% transposition frequency of *Ds* in T0 (Table 3.5; Table A1), when *Ds-GUS* derived calli were bombarded with the *Ac*Tpase plasmid (Ubi-*Ac*Tpase-*GFP*). However, a higher remobilization efficiency of 16.9 % was observed in the T1 population generated by self-pollination of lines containing both *Ac* & *Ds* elements (Table 3.5).

Moreover, empty donor (ED) analysis was applied for both self-pollination and transient-based populations, to confirm the *Ds* movement. In the case of population generated by *Ac*/*Ds* self-pollination, the presence of the ~490bp amplicon indicated the excision of *Ds*Ubi-*Bar* from its original backbone (Figure 3.22A). We chose one parental line T0 (OT5), and two *Ds* T1 lines (OT5-44, 48), which were derived from the self-pollination of this parental line. Empty donor amplicon bands were isolated, purified, and sequenced. Sequencing results revealed a large deletion and genome modifications (119 bp for 5' side, and 146 bp from 3' side) around *Ds*-TIRs after transposition (Figure 3.23A). This footprint-based deletion was observed in the parental line T0 (OT5), and inherited in T1 (OT5-44, 48) derived from this parental line. In contrast, in the case of the transient *Ds*-activation population, screening of 63 plantlets (Table A1) showed a very high excision frequency (74.6%), based on empty donor PCR analysis. Examples of these ED amplicons are shown in Figure 3.22B. Sequencing these amplicons showed large deletions of (308 bp on 5´ side and 1056 bp on 3´ side), in addition to restructuring in *Ds* adjacent sequences (Figure 3.23B).

Table 3.5: Primary and secondary transposition obtained by bombardment or co-bombardment using several Maize *Ac/Ds* constructs, including the different techniques used for generation of the populations, parental lines, number of plants screened, the number of the transposition events obtained and the transposition frequency.

Transposition Activation conditions	***Ac/Ds* Parental lines (T0)**	**Plants tested (T1)**	**Transposition Events (OTPs)**	**Transposition Frequency %**
Tissue culture stage	All OTs	20	1	5
Generation advance of oat transposon lines containing both *Ac/Ds* elements	OT1	60	9	15
	OT4	89	15	16.85
	OT5	89	6	6.74
	OT8	12	-	-
	OT11	13	-	-
	OT12	15	-	-
Transient *Ac*Tpase shock	OT22	63	10	15. 87

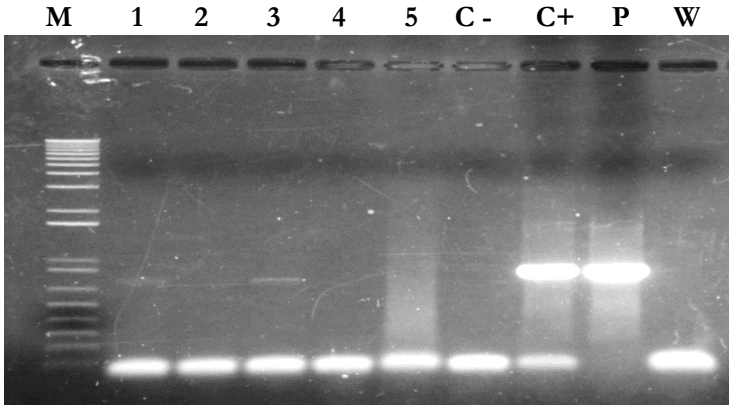


Figure 3.20 Transcript abundance of *Ac*Tpase in leaves of T0 oat transgenic lines. The bands in lanes 1 and 3 indicate the specific amplicon expected using AC3, AC5 primers, respectively. The Ubi-*Ac*Tpase plasmid (P) and gDNA of the same sample as in lane 1 (C+) were used as positive controls, while non-transgenic oat (C-) served as a negative control in addition to the water (W). (M) is 1kb plus DNA marker.

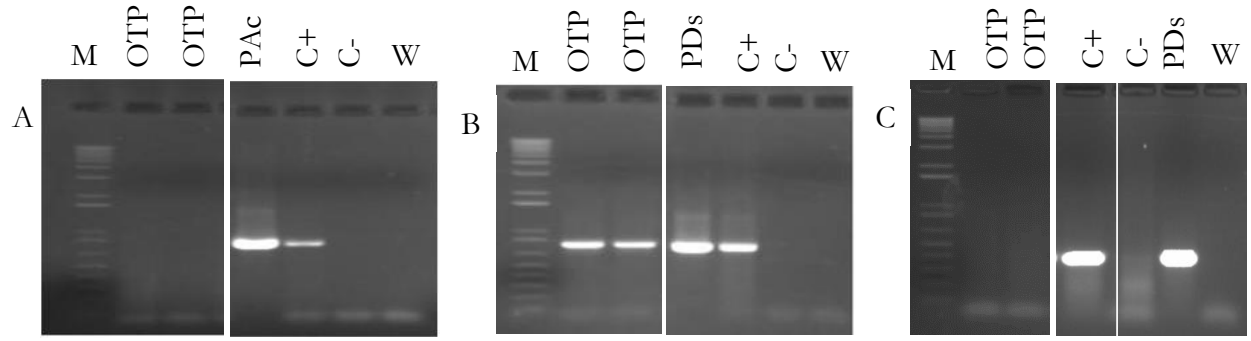


Figure 3.21 Molecular screening for *Ds* transposition events. (**A**). PCR analysis to identify the presence of Ac element in the selected transplants, using Ac transposon-specific primers, amplicon size ~825 bp. The absence of this amplicon in OTPs lanes indicates the absence of the Ac element in these events. As positive controls, we used Ubi*Ac*Tpase plasmid, and Ac transgenic line (C+). (**B**) PCR analysis to identify the presence of *Ds* element in the selected transplants, using Ds transposon-specific primers, amplicon size ~813 bp. Bands (OTPs) indicate the presence of the Ds element in these events. As positive controls, we used the *Ds*Ubi-*Bar* plasmid and *Ds* transgenic line (C+). (**C**) PCR analysis for identifying the transposition events of the Ds element. Using two primers, forward one starts the transcription from the 3´TIR of *Ds* element (JIPF1), and reverse primer starts the transcription from the construct backbone (JEDS3), with an amplicon size of ~444 bp. The absence of the expected product size in the lines (OTPs) indicates the putative jumping event. As positive controls, we used the *Ds*Ubi-*Bar* plasmid and *Ds* transgenic line (C+). For the three PCRs we used non-transgenic line (C-), and water (W) as negative controls, while (M) is 1kb plus DNA marker.

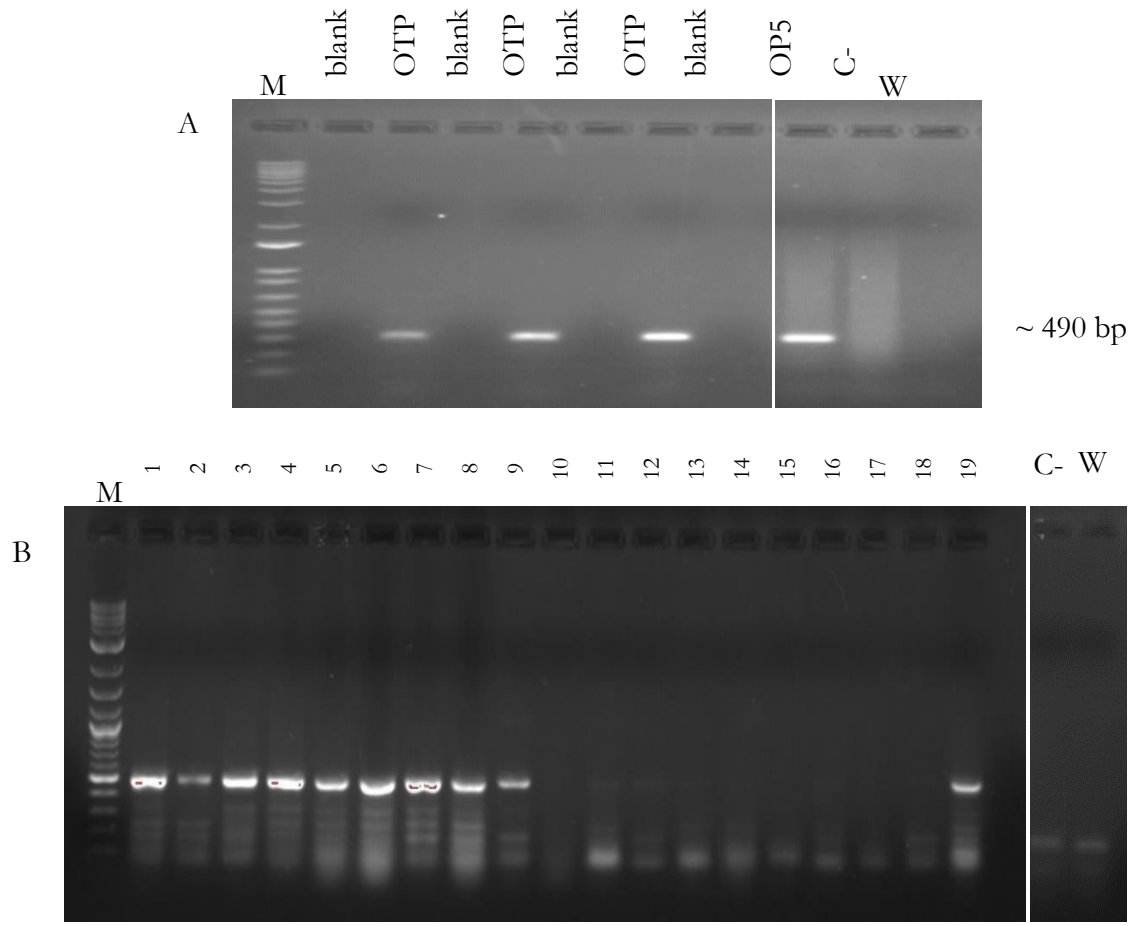


Figure 3.22 A Empty Donor (ED) PCR analysis. **(A)** Represents the (ED) amplicons observed in the *Ds*Ubi*Bar* population. Specific primers (EDs5, EDs3) from the *Ds*Ubi*Bar* construct backbone, adjacent to both *Ds*5 and *Ds*3 boarders were used. Obtaining an amplicon indicates the excision of *Ds* from its original site, and hence its remobilization. (**B**) Represents the ED amplicons observed in the *DsBarGus* population generated by the transient *Ds* reactivation assay. Primers (Vec8-5-F2, and UbiDs-UidAR2) were used to amplify the ED site. Primers are matching specific sequences flanking *Ds* sequences in the *Ds*Ubi*GUS* construct. Bands observed in lanes 1 to 9, and 19 represents the ED footprint in these lines. These lines correspond to the lines (OTv38-46, and OTv 56), respectively (Table A2). As negative controls, we used a non-transgenic oat line (C-), and water (W), while (M) is 1kb plus DNA marker.

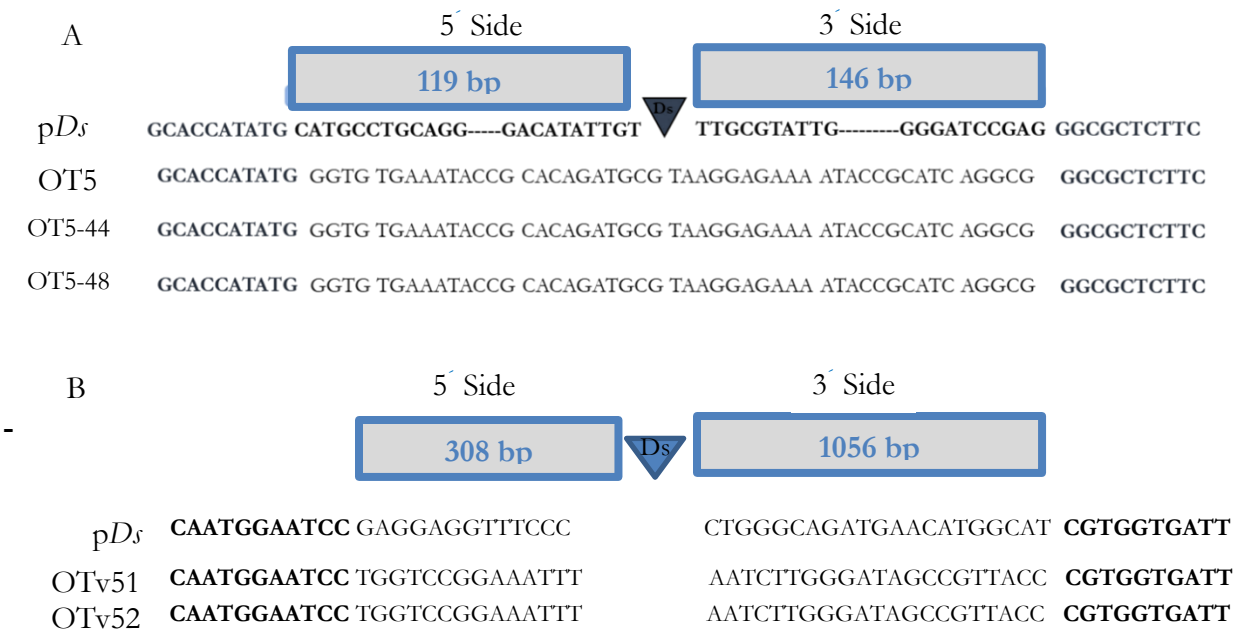


Figure 3.23 The *Ds* launch site structure (Empty Donor sites). **(A)** Represents the footprint of the *Ds* excision site left in the *Ds*Ubi*Bar* construct. pDs indicate the original *Ds* insertion site, where the *Ds* is represented by a triangle, and both (119 bp at 5´side and 146 bp at 3´side sequences) are presented in the *Ds*Ubi-*Bar* construct and deleted in the OT line, after *Ds* excision in the parental T0 line (OT5), and its T1 progeny lines (44, 48), respectively. **(B)** Represents the footprint of the *Ds* excision site left in *Ds-BarGUS* after the transient reactivation assay. The *Ds* (triangle) indicates the original insertion of *Ds* in its plasmid *DsBarGUS* (p*Ds*). OTv51 and 52 represent examples of footprints isolated from the population generated by the transient reactivation assay, where large deletions (308 bp at 5´side and 1056 bp at 3´side) were observed.

3.4.8. TaqMan qPCR for copy number variation (CNV) in transgenic oat

Copy number was estimated for transgenic oat containing the *Bar*-selectable marker. This marker is located between the two *Ds* inverted repeats, in both *Ds*Ubi*Bar*, and *DsBarGus* constructs. Thus, the copy number of the *Bar* gene is an indicator of the CNV of *Ds* in the tested plant. A total of 14 *Ac*/*Ds* T0 transgenic lines (OTs), and 4 T1 transposition lines (OTPs) were screened (Table 3.6). For calculation of the CNV of *Bar* gene, genomic DNA isolated from each oat plant was run, in triplicate, in a single-plex TaqMan assay. The Ct value of the calibrator (Single copy barley *Ds*Ubi*Bar* line -TNP-280) and oat transgene lines were first normalized by comparing them to the Ct value of the reference gene (acc1), and then comparing them to each other. As explained in the method section, the copy number was calculated as CNc = (2 – ΔΔCt), and estimated following the criteria (CNe = 1, 2, 3, or a higher copy number the calculated CNe was less than 1, 1 to 2, 2 to 3, and over three, respectively). Based on our results, 93% of transposon transgenic lines (T0) showed a single copy insertion, and only 7.1% showed two copies of *Ds* (Table 3.7). All four transposition lines (OTP13, 16, 21, and 26)

showed a single copy of *Ds*, which was expected due to the movement and segregation from other copies, a unique feature of the *Ac/Ds* system. Low copy number were also projected in other lines as both Ac and *Ds* containing plasmids were co-bombarded allowing movement and segregation at the T0 stage. This observation has been also made previously in a barley *Ac/Ds* system (Koprek et al. 2000).

Table 3.6. TaqMan assay for copy number variation in transgenic *Ac/Ds* oat lines

Oat Transposons lines	Gene-ration	Average Ct of endogenous control (*acc1*) (Cta)	Average Ct of target gene (*Bar*) (Ctb)	[1]ΔCt	[2]ΔΔCt	[3]CNc calculated	[4]CNe estimated
OT1	T0	25.97667	22.44667	-3.53	-0.17667	1.130269	2
OT2	T0	24.83333	26.11333	1.28	4.633333	0.040293	1
OT3	T0	23.45667	27.73	4.273333	7.626667	0.00506	1
OT4	T0	25.85667	27.96	2.103333	5.456667	0.022771	1
OT5	T0	23.80667	24.77333	0.966667	4.32	0.050067	1
OT6	T0	23.76333	24.99333	1.23	4.583333	0.041714	1
OT7	T0	26.97333	25.54333	-1.43	1.923333	0.263645	1
OT9	T0	25.54333	31.19333	5.65	9.003333	0.001949	1
OT10	T0	26.20667	26.36667	0.16	3.513333	0.087575	1
OT13	T0	24.56	26.56333	2.003333	5.356667	0.024405	1
OT15	T0	27.06333	27.3	0.236667	3.59	0.083043	1
OT16	T0	26.49667	25.49	-1.00667	2.346667	0.1966	1
OT17	T0	25.35	30.02333	4.673333	8.026667	0.003835	1
OT22	T0	25.93	25.55333	-0.37667	2.976667	0.127038	1
OTP16	T1	29.25667	30.74667	1.49	4.843333	0.034835	1
OTP21	T1	29.97333	31.06333	1.09	4.443333	0.045965	1
OTP26	T1	27.38333	30.48667	3.103333	6.456667	0.011385	1
OTP13	T1	25.63	29.05333	3.423333	6.776667	0.009121	1
Calibrator	T1	29.86333	26.51	-3.35333			

[1]ΔCt= Ctb*Bar* - Cta*acc1*
[2]ΔΔCt=Δt Sample –ΔCt Calibrator
[3]CNc, calculated copy number = $(2^{-\Delta\Delta Ct})$
[4]CNe, estimated copy number, following the criteria;
1 copy, if CNc is less 1
2 copies, if CNc between 1 to 2
3 copies, if CNc is between 2 to 3
High copy number, if CNc over than 3

Table 3.7. The copy number frequency estimated based on TaqMan assay, of T0 oat *Ac*/*Ds* transgenic lines.

Estimated copy number CNe	N° of *Ac*/*Ds* (T0) lines tested	Estimated CN frequency
1 copy	13	92.87%
2 copies	1	7.14%

3.4.9. Identification of the *Ds* alternative insertion sites

Different types of mutations can occurred as a result of the remobilization of heterologous *Ds* elements in the oat genome. These mutations vary based on the insertion site, and the modified *Ds* element used in our construct, either knocking out or activating the tagging construct. Identification of the transposition insertion sites is essential in reverse functional genomic systems. Thus, *Ds* transposition events were subjected to further *Ds* flanking sequence isolation techniques. These methods include Inverse PCR, HE-TAIL PCR, and Adapter ligated method ALPCR (Gene Walker). The latter, as an example (Figure 3.24) shows the bands obtained with Gene Walker kit (ALPCR) applied to several transposition events.

A total of twenty-one transposition lines were subjected to the isolation of *Ds* flanking sequences using various techniques described above. Isolated fragments were sequenced at the Génome Québec Innovation Centre, McGill University (examples of these sequences are shown in Table A1). Four transposition lines (OTP11, 23a, 23b, and 35) showed unique *Ds* insertion sites (sequences provided in supplementary Table 3.8, Appendix). Using the *Avena* genome database (https://avenagenome.org/), three lines (OTP11, 23a, and 23b) were found to show *Ds* insertion in chromosomal sites with significantly homology to three *Avena* raw sequences (CTG47356, CTG34937, and CTG6510), respectively (Table 3.8). However, no functional genetic information regarding these three alignments is yet available. Moreover, using the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to annotate these flanking sequences, only the OTP23b transposition line revealed a *Ds* insertion in one sequence which has homology to a functionally identified gene (GA20ox3), with an e value of 2×10^{-13} in barley (Table 3.9). In the rest of the sequences, the *Ds* sequence was lacking, which was probably due to the location of primers near the end of *Ds* sequence (in *Ds*Ubi-*Bar* cassette). Further experiments are required to confirm of the presence of *Ds* flanking sequences in other lines with transposition events.

Table 3.8 Summary of *Ds* alternative insertion site mapping in oat transposition lines, including methods used for isolation the flanking sequence, size of sequence isolated, cereal sequences, and accessions matched, and *e* value. Results were obtained using the *Avena* Genome database (https://avenagenome.org/).

OTPs	Method	Size (bp)	Side	Sequences alignments (*Avena* Genome Project) Database	e value
OTP11	Gene walker kit	1906	5	CTG47356	0.00
OTP26a	Inverse PCR	536	5	CTG34937	0.00
OTP26b	Inverse PCR	172	3	CTG6510	1.32×10^{-32}
OTP34	Gene walker kit	420	5	None	

Table 3.9 Summary of *Ds* alternative insertion site mapping in oat transposition lines, including methods used for isolation the flanking sequence, size of sequence isolated, cereal sequences, and accessions matched, and e value. Results were obtained using NCBI database https://blast.ncbi.nlm.nih.gov/Blast.cgi

OTPs	Method	Size (bp)	end	Homology	Gene bank Accession	*e* value
OTP11	Gene walker kit	1906	5	None	-	-
OTP26a	Gene walker kit	365	5	None	-	-
OTP26b	Inverse PCR	536	5	*Hordeum vulgare* GA 20-oxidase 3 (GA20ox3)	AY551429.1	2×10^{-13}
OTP34	Gene walker kit	420	5	None	-	-

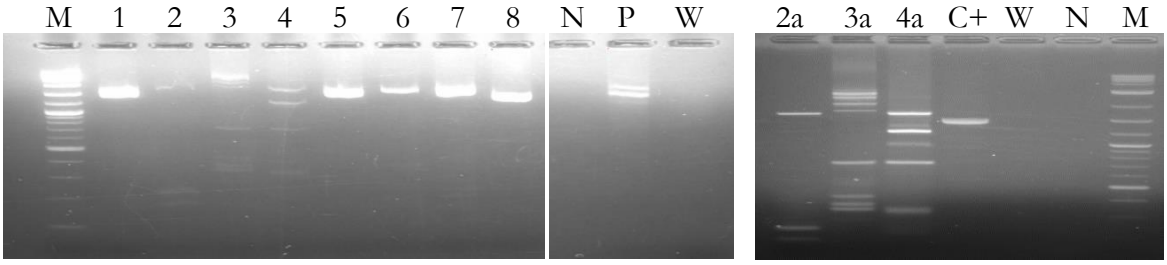


Figure 3.24 An example of PCR products obtained by the Adapter ligation method (Universal gene walker kit). In order to identify the *Ds* insertion site, genome DNA was digested, adapters ligated and then amplified for each transposition line. PCR products of lines 1, 5, 6, 7, and 8 were purified directly, while PCR products of lines b, c, and d were re-amplified, run on a gel (1.6% agarose), shown as 2a, 3a, and 4a, and the multiple bands cut and purified. The bands were purified and sequenced. (M, N, C+, and W) represent 1Kb plus a marker, non-transgenic oat as a negative control, a human genome positive control, and water, respectively.

3.4.10. Phenotypic observations

Three phenotypes (OTP14, 16, and 24) were observed when the population of T1 plants was screened. These phenotypes were associated with stable *Ds* transposition events in a T1 population resulting from a *Ds*Ubi-*Bar* & Ubi-*Ac*Tpase self-pollination population. These three events exhibited similar phenotypic patterns: warped leaves, and growth abnormalities, which resulted shorter plant (Figure 3.25-T1). OTP16 was infertile, while both OTP14, and 24 produced few seeds. To explore the inheritance of this phenotype, second-generation T2 were generated from both fertile lines. Due to the low number of seeds obtained, only three T2 plants were generated from each T1 line. Interestingly, one (OTP14 A) of three T2 plants showed a similar phenotypic pattern (short plant, and warped leaves), coupled with loss of fertility (Figure 3.25-T2). The co-inheritance of the *Ds*-phenotype through the two generations indicates a possible link between the *Ds* insertion and the phenotype observed. Further investigations are required to explore the insertion site of *Ds*, and the correlation between this insertion and phenotype obtained.

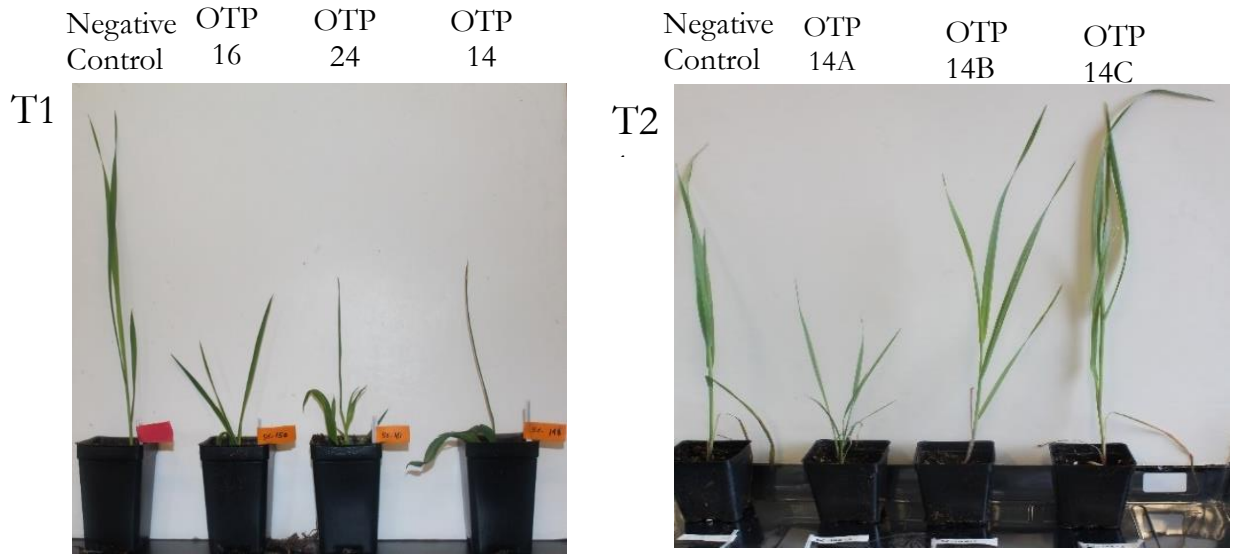


Figure 3.25 Observation of phenotypes in the Ubi-*Ac*Tpase /*Ds*Ubi-*Bar* population. OTP14, 16, 24 represent plants from T1 generation where this phenotype was observed. OTP14A, B, C are T2 plants derived from OTP14. An oat non-transgenic line is used as a negative control.

3.5. Discussion

Introduced as one of the most important functional food for human consumption, the common oat has drawn increasing interest after the discovery of its healthy components (Abuajah, 2017; Nosrati et al., 2017). However, a lack of adequate oat functional genetic and genomic information retards urgently needed breeding programs for the improvement of common oat. In conjunction with revolutionary sequencing techniques (e.g., genotyping by sequencing, GBS), employing reverse genetic approaches is essential to forwarding the exploration of the functional roles of identified genes. Among these methods, a maize *Ac*/*Ds* transposons approach, successful in reverse genetic mutagenesis in cereals (Singh et al. 2006; Ayliffe et al. 2007; Ito et al. 2002; Taylor and Walbot 1985; Wilson et al. 1996; Jeong et al. 2002), was examined. This system consists of two elements: *Ac*, the coding sequence for the *Ac*Tpase enzyme and a modified *Ds* element. *Ac*Tpase can identify the two terminal inverted repeats of the *Ds* element, and then induce double-strand DNA breaks (DSBs). The excision of the *Ds* element is followed by the insertion of this element in an alternative chromosomal site. This mechanism is known as cut and paste transposition (Goodwin et al. 2003).

In the present research, we were the first to introduce and investigate heterologous *Ac*/*Ds* transposons in oat. Developing such a heterologous transposons system in oat provides a valuable resource for functional genetic identification, which can be drawn upon in further oat breeding programs.

Firstly, molecular screening-based PCRs were performed to detect the presence of homologous native maize Activator (*Ac*) and Dissociation (*Ds*) transposable elements in the oat cultivar 'Park', commonly used in biolistic-based transformation experiments in oat (Somers et al. 1992; Torbert et al. 1995; Torbert et al. 1998; Cho et al. 1999; Kaeppler et al. 2000). These studies reported a high regeneration ability of this genotype under tissue culture conditions. Accordingly, we chose this variety as a source of explants for our genetic transformation experiments.

The *Ds* transposon is an incomplete size sequence derived from the *Ac* element (Fedoroff et al. 1983). The *Ds* terminus and the *Ac*Tpase coding sequence are significant markers for these types of transposable elements. Two primer sets were used to amplify specific sequences from *Ds*5' side, and *Ac* transposase encoding sequence (Table 3.2). No amplicon was observed, which indicates the absence of these two elements in the oat genome (Figure 3.7). We performed further blast searches against the oat genome project database http://www.avenagenome.org/. In particular, three main sequences were subjected to the blasting process, the *Ds3' and Ds5'* inverted repeats, and the *Ac* transposase coding sequences). No significant similarity was found, confirming the absence of both

maize *Ac* and *Ds* transposons in the common oat genome. These results concurred with those of Luo et al. (2012), who was unable to identify *Ac*/*Ds*-type transposons in 319 plasmid clones derived from the common oat 18D chromosome. However, due to the lack of a full sequencing database for common oat, no deep investigations of native *Ac*/*Ds* like elements have been reported in this cereal. Thus, providing further sequencing information may reveal homologous sequences in oat.

After the native *Ac*/*Ds* analysis, we started the transformation process using the Biolistic system. The gene gun (biolistic) technique is broadly used in oat transformation studies (Kaeppler et al. 2000; Cho et al. 1999; Somers et al. 1992; Torbert et al. 1998).

Since the transformation process in cereals is not routine, we first wanted to confirm the activity of this *Ac*/*Ds* system in the oat genome. Thus, transient *Ac*/*Ds* activity was tested in the common oat variety 'Park'. The expression of the *Ac* element catalysis led to the *Ds* excision from its original *Ds-GUS* construct. This excision restores the *GUS* gene expression in the transformed oat calli pieces (OCPs). We co-bombarded two specific *Ac*/*Ds* constructs; (pCambia-ActGFP-Ubi*Ac*) (Figure 3.6) as a source of *Ac* transposase protein, and *Ds-Gus* (Psp-wdv-Act1-*DsBar-GUS*.n) (Figure 3.2) bearing the *GUS* gene interrupted with a *Ds* element (*GUS* Trap). Forty-eight hours later, co-bombarded OCPs were selected randomly and subjected to the *GUS* assay. A blue color reflected the chemical reaction between the beta-glucuronidase protein, encoded by the GUS gene, and the X-Gluc reagent. Positive *GUS* (blue) sections or foci were observed in the tested OCPs (Figure 3.8). This observation confirmed the functional activity of the *Ac* / *Ds* system in the common oat genome. Similarly, transient activity was obtained, for the first time, in cultivated barley (McElroy et al., 1997a), and then in its wild relative (*Hordeum vulgare* ssp. *spontaneum*) (Cardinal et al. 2016).

The previous confirmation analyses were followed with several *Ac*/*Ds* bombardment-experiments, using the same genotype (cv 'Park'). Biolistic transformation experiments were performed following Cho et al. (1999) transformation approach and recommendations.

In our study, we used several different *Ac*/*Ds* constructs for oat transformation (Table 3.3). The main objective of the study was to introduce heterologous *Ac* and *Ds* transposable elements into the oat genome. For this purpose, two different types of transposon constructs were used for the development of functional genomic resources with gene knock out and activation ability. In the case of activation of the tagging plasmid (*DsBar-Gus*) (Figure 3.4), two Ubi promoters were present in the construct in forward and reverse directions, and out of the construct. These promoters can enhance

the transcription of the genes adjacent to the alternative *Ds* insertion site. Due to the activation feature, the vec8 plasmid is known as an activation tagging construct (Ayliffe et al., 2007). Alternatively, the *Ds*Ubi-*Bar* (pSP-*Ds*-Ubi-*Bar*-nos-*Ds)* construct (Figure 3.3) only has a knock-out function. Two different selection mediums were used based on the selection marker included in the bombarded constructs. These mediums include phosphinothricin (bialaphos), and hygromycin at concentrations of 3 mg L^{-1} and 30 mg L^{-1}, respectively. After the selection process, the regenerated plantlets were subjected to histochemical and molecular analysis.

A total of 2035 calli pieces were used as the target of several independent transformation experiments, with four being successful in generating a total of 24 transposon transgenic events. A transformation frequency of up to 9.5% was observed using the bialaphos selection process, higher than the 5% frequency obtained by Gless et al. (1998), using same selection marker in oat. On the other hand, a higher transformation frequency (34%) had been previously obtained in oat using the same selection process (Somers et al. (1992). These results contrast with our analyses, in the latest experiment; however, only one fertile plant was obtained in the study by Sommers et al. (2002), so the integration of the transgenes into the host genome was not investigated.

While using hygromycin selection, a maximum transformation efficiency of 1.9% was obtained, significantly lower than the 25.6% efficiency achieved by Cho et al. (1999) using the same genotype (cv 'Park'), and a similar hygromycin selection process. This could be due to the condition of the calli, and a poor lot of hygromycin as has given spurious results in other transformation experiments (Fatmawati, Kaur and Singh, unpublished).

These transformants (OTs) included two Ubi*Ac*Tpase, two *DsBar-GUS*, thirteen *Ds*Ubi-*Bar* events, in addition to seven events bearing both Ubi-*Ac*Tpase and *Ds*Ubi-Bar elements. Unfortunately, both the Ubi-*Ac*Tpase lines were infertile, and therefore did not produce any seeds. Infertility is a common phenomenon in biolistic transformation in oat. Somers et al. (1992) reported a similar infertility in 97% of plantlets regenerated from bombarded oat calli. Likewise, Cho et al. (1999) reported up to 37% infertility when using the same transformation system in oat.

Screening the progeny of our transformants confirmed the integration of heterologous *Ac*/*Ds* elements in the oat genome, which led to the inheritance of these elements during the generation advance. For example, thirty-one T1 plants, derived from two T0 *DsBar-Gus*-putative transgenic lines, were screened through biochemical and histochemical methods. As a result, 14 of these plants showed

blue foci, indicating a 58% frequency of *Ds-GUS* integration into the oat genome (Figure 3.15). Moreover, our molecular analyses confirmed this integration (Figure 3.16).

The co-bombardment of both *Ac* and *Ds* elements, into the calli pieces, may induce *Ds* transposition activity through the tissue culture stage. Such primary transposition events may occur, but only if *Ac* and *Ds* are co-bombarded or hybridized together and *Ac*Tpase is expressed in the host genome. Firstly, the transcript abundance of *Ac*Tpase was confirmed from two randomly selected lines containing both *Ac* and *Ds* (Figure 13.20). Then, a total of 20 transgenic lines with transposons were subjected to different PCR analyses (Table 3.2). These PCRs were classified in four categories: (i) presence of *Ac*, using specific *Ac*Tpase primers, (*ii*) the presence of *Ds*Ubi-*Bar*, using specific *Ds*Ubi-*Bar* primers, within the *Ds* element, (*iii*) the presence of *Ds*Ubi-*Bar* in its construct, using two primers, forward within *Ds* element, and reverse matches to an adjacent backbone sequence, and (*iv*) *Ds* transposition activity was further confirmed, empty donor PCR (launch pad) was carried out using two primers flanking the 5' and 3' *Ds* elements.

Molecular screening analysis of T0 putative transgenic lines showed one transposition event, indicating a 5% transposition frequency at the calli stage. In this event, no *Ac* was detected, while *Ds* was found to be outside the plasmid backbone. Such a transposition event can have occurred due to a transient expression of *Ac*Tpase. Similar primary *Ds* transposition events, under extrachromosomal expression of *Ac*Tpase, were reported in barley (Singh et al., 2012).

Furthermore, we developed populations for transposition event screening. Two different techniques were used for generating these populations: (*i*) seeds from a *DsBar-GUS* activation tagging transgenic line were used as explants for generation green calli. These calli pieces were then bombarded by gold particles coated by Ubi*Ac*Tpase-GFP construct. After 24 hours, the calli were screened for the GFP expression. Calli with, GFP expression (Figure 3.18), were considered to indicate the synthesis of the transposase protein due to its fusion with GFP, and these were thus selected for regeneration. A total of 63 plantlets were regenerated from the selected calli. Molecular screening of these plants suggested a total of 10 transposants, representing a transposition frequency of 15.87%. This reactivation frequency is lower than that the 34-39% previously reported for barley by Singh et al. (2012). However, in this study, an *Agrobacterium*-mediated transformation system was used. Also, the reactivation of hetreologous transposons could be a species-specific event and also dependent on the construct and promoters used to drive the genetic elements. The developmental reprogramming process may power

such primary transposition frequency during the tissue culture stage. This high regeneration activity is likely associated with an alteration in the methylation process and chromatin structure (Ki et al. 2002). It could also be hypothesized that the reset of the *D*s methylated TIRs is positively correlated with the remobilization activity of the *Ds* element, as *Ac*Tpase may only recognize unmethylated *Ds* TIRs (Smulders and De Klerk 2011; Ros and Kunze 2001).

Secondly, transposons transgenic lines containing both *Ac*Tpase and *Ds*Ubi-*Bar* constructs were self-pollinated. Out of 278 screened seedlings, 31 transposition events were detected, indicating a transposition frequency of up to 16.9% (Table 3.4). These results are consistent with a previous report in barley, in where a frequency range of 5 to 41% was observed in an F2 population (Brown et al. 2015). Moreover, *Ds*-transposition frequencies of 11.8% to 17.1% (Singh et al., 2006), and 10.5% and 9.8% (Singh et al., 2012) were reported in transposon populations created via generation advance. Upadhayaya et al. (2002) reported a somewhat lower *Ds* transposition efficiency of 6.6–11.5% in rice. In contrast, Koprek et al. (2000) showed a high transposition frequency (47%) in barley, but no *Ds* flanking sequences were provided, and thus, many redundant events might have been scored as unique and included in the transposition frequency. A high transposition efficiency (51%) was also achieved by Kolesnik et al. (2004), in rice, but was likely due to either to redundancies or the use of different promoters for *Ac*Tpase expression. Moreover, Kolesnik et al. (2004) used the CaMV 35S promoter to activate the *Ac*Tpase genes, while studies conducted by Upadhayaya et al. (2002) and Singh et al., (2006) used the maize ubiquitin1 promoter to regulate the *Ac*tpase. These variations may have affected the timing and thus, the transposition efficiency of *Ds*, as observed in *Arabidopsis* (Smith et al. 1996).

Furthermore, we explored the copy number variation of *Ds* in a total of fourteen *Ac*/*Ds* T0 lines (OTs), and four T1 transposition lines (OTPs) (Table 3.6). The QPCR reaction and cycling conditions were as in Ingham et al., (2001), but a single-plex assay was employed. Our CNV calculation and estimation is explained in the methods section. As a reference gene, we chose the oat *Acetyl-CoA carboxylase* (*Acc1*) gene. Previously, three copies of the plastid *ACCase* gene (*Acetyl-CoA carboxylase or Acc1*) had been reported by Gornicki and his colleagues (1997) in bread wheat (*Triticum aestivum*), (single copy in each chromosome set). This gene's stability was later confirmed by Hernández et al., (2005). This study recommended *acc1* as a preferred reference gene for copy number variation of transformation-based biolistic in bread wheat. In common oat, the orthologue of wheat *acc1* was first cloned by Kianian et al., (1999), and entered into the NCBI database, under accession number

(AF072737.1). Like bread wheat *acc1*, oat *acc1* is also a single copy gene, and so it has been used in the phylogenetic analysis of different *Avena* species, including the common oat (Yan et al., 2014). Thus, the oat *Acetyl-CoA carboxylase* (*Acc1*) gene was chosen for use in our study, to serve as an endogenous reference gene for the TaqMan assay in oat. We chose the TNP-280 line as a calibrator, namely a barley mutant carrying a *Ds* single copy insertion.

The TaqMan assay showed a single or low copy number in almost all the T0 *Ac*/*Ds* lines tested (Table 3.7). A single copy of *Ds* was observed in four transposition lines (OTP13, 16, 21, and 26). Some previous studies using the same biolistic-transformation system have reported a high copy number for transgenes (Pawlowski and Somers 1996; Kohli et al. 1998; Choi et al. 2000; Darbani et al. 2008; Gasparis 2017). However, in these studies, different transposable elements were introduced individually, whereas in our study co-bombardment of the *Ac*/*Ds* system enabled the *Ds* element to transpose and segregate away from other copies. This unique feature explains the single low or single copy present in our lines. For example, using the biolistic system, Singh et al. (2006) showed a single and low copy number of *Ds* in four *Ac*/*Ds* successive generations (T0, T1, T2, and T3) in barley. In contrast, in wheat, an average of 44.8% single copy insertions out of the total transformants was obtained using the biolistic system (Ismagul et al. 2018).

The movement of *Ds* outside its original site often leaves an alteration behind, which is considered as an important indicator of this transposition process. This alteration occurs due to repair mechanisms errors. Using empty donor PCR, we detected this footprint in several lines. For instance, the OT5 T0 line showed a footprint where deletions of 119 bp from the 5′ side, and 146 bp from the 3′ side were observed around *Ds*-TIRs in the plasmid *Ds*Ubi*Bar* launch site (Figure 3.23A). Moreover, the transient activation-derived population was also screened, using empty donor (ED) PCR, for excision footprints and their frequency. Our data indicate a very high *Ds* excision frequency (74.6%), an example of the ED analysis being shown in Figure 3.22B. This excision was confirmed by sequencing the footprint resulted from to the *Ds* movement. Interestingly, sequencing data showed sequence-restructuring and large deletions of 308 bp, and 1056 bp from 5′ and 3′ sides, respectively, around *Ds*-TIRs, in the plasmid *DsBarGus* launch site (Figure 3.23B). More investigations are required to explore whether this sequence-restructuring is generated due to the *Ds* remobilization, or biolistic operations. Such large deletions were previously reported in *Arabidopsis* (Page et al., 2004). In this study, deletions of up to 104 kb were observed in chromosomal sites adjacent to *Ds* donor launch sites. This analysis provides

incidence of transposon activity in lines containing more than one *Ds* copy, where one copy present in the plasmid may mask the results of other transposition copies.

The determination of the *Ds* insertion sites is imperative in any reverse mutagenesis approach for functional gene identification. Therefore, we applied several techniques for *Ds* flanking sequence isolation, including nested Inverse PCR (IPCR), High-TAIL PCR, and Adapter Ligation methods (ALPCR), as well as a gene walking technique (Figure 3.24). Flanking sequences of 21 transposition lines were isolated. Four unique insertion sites showed a presence of the *Ds* sequence. These sequences were annotated against the NCBI database. As a result, one of these *Ds* insertion (OTP23b) was found in the *Hordeum vulgare* GA 20-oxidase 3 (GA20ox3) gene (2×10^{-13}) (Table3.9). Similar preference of *Ds* transposition to genetic regions was reported in barley (Cowperthwaite et al. 2002; Kolesnik et al. 2004; Singh et al. 2006). Moreover, out of these OTPs, three lines (OTP11, 23a, and 23b) showed *Ds* integration in different chromosomal sites sharing homology to three *Avena* raw sequences (CTG47356, CTG34937, and CTG6510, respectively; Table 3.8.

This database provides the accessions names and their complete sequence. However, no functional genetic and genomic information for these accessions has been provided yet. Thus, we could not determine whether the rest of the raw sequences in the *Avena* genome database https://avenagenome.org/ were derived from genic regions.

Moreover, a unique phenotype was observed (a smaller plant with warped leaves coupled with a loss of fertility) in three T1 transposition lines (OTP16, 24, and 14). These lines were generated from the OT3 parental line (Figure 3.25-T1). Interestingly, co-inheritance of *Ds* insertion and a similar unique phenotype was also seen in one out of three T2 plants (Figure 3.25-T2). Koprek et al. (2001) similarly reported a unique short plant phenotype resulting from *Ds*-insertion in two *Ac*/*Ds* F2 barley plants. In the latest study, *Ds* was also co-inherited, with the phenotype into F3 progeny. Our phenotype required further investigation to explore the link between the *Ds* insertion and the phenotype generated.

This is the first ever successful effort in developing a transposon-based mutagenesis resource in oat and has the potential to explore the oat genome and provide future applications for both forward and reverse genetic systems for the identification of genes associated with desirable traits.

CHAPTER IV: GENERAL CONCLUSION

The common oat is one of the most cultivated cereals in the world. In the last few decades, the demand for oat as human functional food has risen significantly. This growing interest is basically the result of recent discoveries regarding the healthy properties of several oat compounds, including soluble fiber β-glucan and avenanthramides antioxidants. Further improvement of current oat cultivars is imperative to meet the high demand for oat grain as a whole, and particularly for these healthy compounds. However, limitations in functional genetic information regarding oats is one of the main obstacles facing oat breeding programs. Using reverse genetics-based applications, several important functional genes were identified either in dicots, or monocots species. Among these techniques, the maize *Ac/Ds* transposons system was applied successfully in cereal crops. Several features make this system as one of the most widely applicable mutagenesis approaches in cereals, including reactivation, high mobilization, and the tendency to transpose to genetic regions. Applying such a transposon-mutagenesis system to oat can provide an effective genetic exploration tool in this valuable cereal. As a first step, our preliminary analysis confirmed the absence of native *Ac/Ds* transposons type in common oat. Since the transformation process in cereals is not routine, we tested the functional activation of heterologous transposons in the oat genome. For the first objective, a *Ds* interrupted *GUS* gene construct was co-bombarded with a *GFP*-Ubi *Ac*Tpase construct. The excision of *Ds*, under the expression of *Ac*Tpase, induced the expression of the *GUS* gene. Our histochemical analysis showed a blue color in the bombarded calli, indicating the successful excision of *Ds* under the transient activity of *Ac*Tpase. Hence, we confirmed the activity of the heterologous *Ac/Ds* system in oat. Subsequently, for the second objective, using the Biolistic system, different *Ac/Ds* constructs were bombarded or co-bombarded into green calli derived from mature seeds of the common oat (cv 'Park'). Two selection mediums were used within the transformation-based tissue culture system, either the herbicide bialaphos, or the antibiotic hygromycin B at respective concentrations of 3 mg L^{-1}, and 30 mg L^{-1}. As a result of our transformation experiments, we generated 24 stable *Ac/Ds* transgenic events, which were confirmed by both biological and histochemical analysis. Out of the 24 events, 22 plantlets were fertile, and hence able to produce seeds. Seeds were collected, and the second generation of these events was developed. Our analysis of the second generation confirmed the inheritance of these elements, and hence their integration into the oat genome. However, two of these events were infertile, so no seeds could be obtained. Based on the selection medium used, our results indicate a transformation frequency of 9.52 % using bialaphos, and of up to 1.9 % using Hygromycin B. Accordingly, we recommend using the bialaphos-based selection in the biolistic-based transformation system, for oat.

For the third objective, seeds were collected from the events that contain both *Ac* & *Ds* and planted in various numbers. The parental events T0, and the populations generated (T1) were subjected to a *Ds* transposition screening. Our molecular analysis showed a primary transposition frequency of 5% through tissue culture of T0, up to 15.9 % through the transient activity of *Ac*Tpase, and up to 16. 9 % in secondary transposition frequency in T1. Our empty donor analyses showed the *Ds* excision footprint in T0 and T1 plants, where a large deletion in the launch site was observed. Such deletion confirms the efficiency of the *Ac*/*Ds* system as a mutagenesis approach in oat. Moreover, a very high *Ds* excision frequency (74.6 %) was detected in the T0 population through an extra-chromosomal expression assay of *Ac*Tpase. Although the integration frequency was not explored in this research, the high excision frequency indicates the efficacy of the transient *Ac*Tpase expression system as a *Ds* remobilization tool in oat. Furthermore, large deletions were observed in the *Ds* launchpad site in both *D*sUbi*Bar* and *DsBarGus*. Such deletions are one of the main features that improve the *Ac*/*Ds* efficiency as a mutagenesis approach. Our TaqMan assay showed a single or low copy number variation of *Ds* in T0 *Ac*/*Ds* transgenic and T1 transposition lines. This low copy number is likely due to the remobilization and segregation abilities of *Ds* away from other copies in the genome.

For the fourth objective, the transposition events (OTPs) were further analyzed for *Ds* alternative insertion identification. *Ds* flanking sequences were isolated, using several nested PCR techniques. Blasting against the oat genome database showed up to 4 unique transposition events, where the *Ds* sequence was observed. Three of these insertion sites were significantly homologous to specific *Avena* accessions sequences. However, functional information regarding these accessions is not yet available. Moreover, using the NCBI database, only one event-insertion site (OTP26) showed a similarity to the *Hordeum vulgare* GA 20-oxidase 3 (GA20ox3) gene. Future development of the common oat sequencing database may reveal the unknown *Ds* insertion sites, in this large genome.

In brief, this is the first study to explore the maize *Ac*/*Ds* system activity in the oat genome. We successfully confirmed the functional activity of this system in oat, and developed a transposon-based functional genomic resource, which provides a significant tool for the identification of novel genes associated with dietary fiber, oil content, disease-resistance and improvement of agronomic traits. Such identified genes can be used for future oat breeding programs, particularly since the high demand for its healthy compounds which shape future research directions.

The future direction of this research should include firstly, the characterization of the *Ds* insertion sites, functionally, and more investigations of the phenotype obtained, and secondly, further reactivation of *Ds*

remobilization using our technique to provide more potential *Ds* insertions and so achieve more gene identifications.

Contributions to Science

1-Development of maize *Ac/Ds* transposons system in common oat provides a major milestone for exploration of the oat genome, and for the functional characterization of oat genes.

2- Our transposition events can be used for further direct reactivation of *Ds*, to target neighboring genetic regions.

3- The transposition events and phenotypes obtained can be employed in the further functional characterization of *Ds*-tagged regions.

Appendix

Table A1. Flanking sequences isolated from oat *Ds* transpositions lines. Partial *Ds* sequence is highlighted in blue.

OTP26a	ID|10863098 PCR57_R5_P1714736_088.ab1 Tgagacggagaggattttacgaccgttaccgaccgttttcatccctagatgagttacagaaaacttacgcgctgacaaggact aaatctgacgtggaggctagactaaaaaaaggaaattacgtgccgctaattttgcaaccaaaagctcaaaccgcccgtacgtt ttgcctcttcgttaaaatcaagcacatcgatctgcctccatcgcgcttggcgtgcagcaggtcgacgcggccgcctgcggccaac acctagccgttccgtcacctgccacaaccaactagccacgccgccgccgccgcctacgaccaacatcttgttcaaactGccctgt acgttttgcctctctgttgatacgagcacaccgagctgcctccattgtgccgtcgcgatcgggactcgtgtacctagctcaccatct Tcctcagcccgcaggtagacctctccgttgagccgccggccgagctgctcgccggatgcccggacgtgccgcaaattatgccaa aaactgcacagtaagttctgttattgttcagatcaaggaagaccactttgtct
OTP26b	Ttttccgacagaacccatgggtaaacccgcagccctaacaccgaatcgagaagaaacacagaaaccccaatggtaaaacaacg cagccctaacaccgaatcgagaagaaactcagaaaccccaatggtaaaacgattggcatgctgctgtctcaccttccccagact ggctgcgccgccgcctacagaatatgtgtcctccatcggctcacctccccctccccctttgggccgcggctccTgcttgcgcttccgcc atgattgaatctcccaagcttctgcttgactggttagtgcttaggccacgtttaatgctgagcgcttgtttaggcgcttgAgcgggaga aacgtacggaagttaatcctattagcgtctaggataggagtccatgcctccgacgcaggctctccagtttagtttatagcgcttctttc ccaaccgaaaatgctaaacctacggatgagttcagggatgaaagtatgatgggaaaatcccgtaccgaccgttatcgtataaccgat tttgttaattttatcccg
OTP11	>ID|11092846 A_P1916188_015.ab1 Cacttaaaacgtacagtattttaccgaccgttaccgaccgttttcatccctacccacatgagtactagagagagaacgag aattcgtgagtggttaaaaaaagaccacaaccataatatgtgcaattagtattgacttttctcaacaaagggtacttccgttaaactgt atgttggaatcaatacaatacaagaaaatagggttcatgtccggcctccggatatacaagatgcacgcaaccgataaaagaggaaaacatagt aaaagggaaaatgcatattcacacacgacgaatatggtggtatgctaacacaaaacgactgtttttttgtaaaagtaaacaactgcgg acgcggtggtggcaatcacatatggtgtcttgatctcgacattgctgttcaaaaccaaagccattagacgagaagaagggtccTaggt ggctaaggttaacgtctagactgatgcagatgtccaattgctccttgccccacaagccgtctttcccaatgaaaatgtgccaatatgtga caagattcccacgatcaagaaggctcgttgccatcatattcaaaggacaaataaataacaaagcagccccctttttttatatctccttttt cttccaagtgatgagtcggttgggacaacaacgcanaagcttggaatgtcactacccaattaataacacttcttaatatacatttTgga ggagtatctattgatagccagggtatagaagtcccatttctctcactaatttaactaacatgagaccctagactccctagtgcaaatgag acaacgagttgcggatgctaagaaagaaaaatgtcaaaccccattagaatcagtagtacattgtgtgcatcaattcgatgcaggaacc gggagggcaagggttaagctctttcttcatcttactaagtaaaaatagcaatgaaaggaagacctttgggcagtggtgtaacccccca
OTP34	>ID|11092559 J91_P1916188_061.ab1 **Ccctagtggtcggtgaggtattttccgaccgttccgaccgttttcatccctatgtcgacgtctagagtcgtcctgcaggcgtg caagcttcagaccagcccgggccgtcgaccacggggtgccctatagtacntcgctcctctctgcaattccccaatgttgcggGgcct ctccctctcctgct
* OTP14	>ID|11092546 D3_P1916188_045.ab1 from 26 to 215 Gaccgttaccgaccgttttctccctacccacatgagtctagagagagaacgagaattcgtgagtggttaaaaaaagaccacaaccataa tatgtgcaattagtattgacttttctcaacaaagggtacttccgttaaactgtatgttggtatcaatacaatacaagaaaatagggttcat gtccggcctc
* OTP12	>ID|11092531 B1_P1916188_095.ab1 Ggacgagtcctttagctttataatgatgttttgtcgctctacatgattatggccattattgctctcttagtttgtcgctcccagtcttttgcta gcctccacaaccaagtatgagttccactcatgcatccaactccctaaaccaagtaattccaaagagtccaccagcccgggccgtcgac cacgcgtgcactatagtaaa
* OTP16	>ID|11004033 TA_f_P1815654_035.ab1 Tctcttctcctgctgtgggccacgtaccaaaagaaaataatcaaaacgctaacaactacaaactctaataacttatagttataggttc

	caaaaaaatagaacccctacaaatacaaactcagaccataaataaatgtatcaagataacatgtgcgtacaattcaaacacttctcct ttttgacaagattcaaatgaaaatcttctttgctcttactacacaaaatctctatagcctaatgtgtagtgtccaatacccccctcctcccc tttttgggaaaaaaaagaatacccccctcctccccctgccccaacatgcacaattttcttcccctttccggaaaaacacatgtttgccaa agtacttcttcccctttccttttcttgatcgcactgattttccattgagctgcatcgtcaatcttattacacctttgatgagcttcttgagatccc tttatgcggctcaaaaagaaaagaaaagaaaatctattgtaaattccaccatttcaactttccagaatggttccggttgtactaccgcataa
* OTP27	>ID|10950492 57H3_P1815352_050.ab1 Aaaacggtctgcggtcttattcgtgcttgcatggttccttggtaacctctagtttgatcagggaaactactgaaaatgaatctgctaat gagggttacaaatttggtcaagaggaagagacttataatattgtggctgctcatggttatttcggccgattaatggatggcttgcctg
* OTP33	>ID|11092544 J8_P1916188_043.ab1 from 43 to 898 Tccctagaaccaggcaactgcaatttgtaaaatgtaagcatattaagctttacctttgcttacccgagggtggaagaacatgagaagg agcacatacaaacacaaagaagtccataatggaggcgtagtccagggagacggtgaggtagccctcttcatcgcgtgcagcag
* OTP 35	>ID|10950493 64D1_P1815352_083.ab1 Ccccgatttgatgctcagggtgaacaaattcaagttgtttttcattcctttggcgcaagcgacttggaggaagcgtcatcggcaataggа gaaccaccctcgaagtcggcggtgtgaagggactcataggagctttggttgccactatcaatggtattcatcggcacgaagacgttgacg tctaataactcggcaa

* *Ds* sequences were not found

Table A2. Molecular screening results of *Ds* movement in 63 T0 lines (OTs) generated using the transient *Ac* Transposase activity. The presence of the (empty donor amplicon) indicates the excision footprint left due to the *Ds* remobilization out of the *DsBarGus* construct, while the presence of the flanking PCR amplicon indicates to the presence of *Ds* in its *DsBarGus* construct and vice versa. Primers used are shown in Table 3.2.

Oat T0 lines	Empty donor PCR	Flanking PCR-3′ side	Oat T0 lines	empty donor PCR	Flanking PCR-3′ side
OTv1	+	+	OTv34	+	+
OTv2	+	+	OTv35	+	+
OTv3	+	+	OTv36	+	_
OTv4	+	+	OTv37	+	+
OTv5	+	+	OTv38	+	+
OTv6	+	+	OTv39	+	+
OTv7	+	+	OTv40	+	+
OTv8	+	+	OTv41	+	+
OTv9	+	+	OTv42	+	_
OTv10	+	+	OTv43	+	+
OTv11	_	+	OTv44	+	_
OTv12	+	_	OTv45	+	_
OTv13	+	_	OTv46	+	+
OTv14	+	_	OTv47	_	_
OTv15	+	+	OTv48	_	+
OTv16	+	+	OTv49	_	+
OTv17	+	+	OTv50	_	+
OTv18	+	+	OTv51	_	+
OTv19	+	+	OTv52	_	+
OTv20	+	_	OTv53	_	_
OTv21	+	_	OTv54	_	+
OTv22	+	_	OTv55	_	+
OTv23	+	+	OTv56	+	+
OTv24	+	+	OTv57	_	+
OTv25	+	_	OTv58	_	+
OTv26	+	+	OTv59	_	_
OTv27	+	+	OTv60	_	_
OTv28	+	_	OTv61	_	_
OTv29	+	+	OTv62	+	+
OTv30	+	+	OTv63	_	+
OTv31	+	_			
OTv32	+	_			
OTv33	+	_			

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