DEVELOPMENT OF FUNCTIONAL GENOMIC RESOURCES IN OAT FOR IDENTIFICATION OF GENES ASSOCIATED WITH BETA-GLUCAN

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Abstract

Oat is one of the major cereals that is grown worldwide for human food and animal feed. Its use in the human diet is becoming popular due to its ability to reduce serum cholesterol and glucose level. This has been attributed to its high β-glucan content. β-glucan is a major non-starch polysaccharide, consisting of double β-1,3 and β-1,4 linkages. Information about β-glucan synthesis in oat is lacking due to the availability of limited genomic resources. Recently, a *Thaumatin Like Protein 8* (*TLP8)* has been identified in barley that interacts with β-glucan and regulates its grain content. We hypothesize that the downregulation of *TLP8* could increase the β-glucan content in oat. The oat *TLP8* ortholog was retrieved, and an RNAi construct was created. Using the particle gene gun bombardment method, genetic transformation was conducted, and transformants were generated using *Bialaphos Resistance* (*Bar*) gene as a selectable marker. This yielded a high putative calli frequency (49.8%) with 7.4% plant transformation efficiency. Histochemical assays confirmed the expression of *Bar,* and transgenic plants were found to be resistant to glufosinate (LIBERTY 0.2%). Expression analysis conducted in T2 RNAi transgenic seeds demonstrated that *TLP8* expression is lower than non-transgenic seeds. In addition, the maize *Ac* and *Ds* transposable elements were successfully introduced into the high β-glucan diploid oat (*A. Strigosa*) to create a transposon-mediated for exploration of other genes associated with β-glucan content. This tissue culture and transformation method in *A. strigosa* is the first of its kind and established a high putative calli frequency (42.8%) with up to 66.6% plant transformation efficiency. To conclude, the genomic resources developed in this study could lead to a better understanding of β-glucan synthesis and regulation in oat.

Resume

L'avoine est l'une des principales céréales cultivées dans le monde pour l'alimentation humaine et animale. Son utilisation dans l'alimentation humaine est devenue populaire en raison de sa capacité à réduire le taux de glucose et cholestérol sérique. Cela est attribué à sa teneur élevée en β-glucane. Le β-glucane est un polysaccharide non-amidon majeur, constitué de doubles liaisons β-1,3 et β-1,4. Les mécanismes de la synthèse du β-glucane dans l'avoine font défaut en raison de la disponibilité de ressources génomiques limitées. Récemment, une protéine de type thaumatine (TLP8), a été identifiée dans l'orge. Cette protéine interagit avec le β-glucane et régule sa teneur dans l'avoine. Nous émettons l'hypothèse qu'un abaissement de l'expression de TLP8 pourrait augmenter la teneur en β-glucane de l'avoine. L'homologue de *TLP8* dans l'avoine a été récupéré et une construction ARNi a été créée. La transformation génétique a ensuite été réalisée via la méthode de bombardement. Les transformants ont été générés en utilisant le gène de *résistance au Bialaphos* (*Bar*) comme marqueur sélectionnable, donnant une régénération élevée avec une efficacité de transformation d'environ 7.4%. Des analyses histochimiques ont confirmé l'expression de *Bar*, et les plantes transgéniques se sont révélées résistantes au glufosinate (LIBERTY 0,2%). L'analyse d'expression menée dans des graines transgéniques T2 ARNi a démontré que l'expression de *TLP8* est inférieure à celle des graines non transgéniques. De plus, les éléments transposables *Ac* et *Ds* du maïs ont été introduits dans l'avoine diploïde (*A. Strigosa*) avec l'intention de créer une ressource génomique fonctionnelle médiée par un transposon pour identifier les gènes associés à la synthèse et à la régulation du β-glucane Cette méthode de culture et de transformation tissulaire chez A. strigosa est la première du genre et a établi une régénération élevée avec jusqu'à 66.6% d'efficacité de transformation. Pour conclure, les ressources génomiques développées dans cette étude pourraient conduire à une meilleure compréhension de la synthèse et de la régulation des β-glucanes dans l'avoine.

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Preface

This thesis is written and designed according to the "McGill Thesis Guideline" (https://www.mcgill.ca/gps/thesis/thesis-guidelines). All thesis components are original work, and the candidate is the first author of these studies.

Contribution of Authors

Dr. Jaswinder Singh and Annis Fatmawati designed the experiments. Annis Fatmawati mainly conducted experiments and analysis. Wei-Yuan Chen contributed to the RNAi construct design. Dr. Rajvinder Kaur contributed to the transformation work. Annis Fatmawati and Dr. Jaswinder Singh wrote the manuscript.

CHAPTER I: GENERAL INTRODUCTION

1.1 Introduction

Oat is an important crop worldwide and one of the major cereal crops which is widely known to lower the blood cholesterol, prevent cardiovascular disease, obesity, and diabetes, and help against coeliac disease, mainly due to its (1,3, 1,4)-β-D-Glucan (β-glucans) composition (Anttila *et al.*, 2004; Butt *et al.*, 2008; Whitehead *et al.*, 2014). Oat places the sixth in production with a total production of 22.51 million metric tons globally in 2019 (USDA, 2019). Oat is mainly used for livestock feed, human consumption, healing agent, particular horse intake, and forage (Webster & Wood, 2011). In the current years, oat demand for food consumption has been significantly surged due to its multifunctional characteristics and nutritional profile in the whole grains (Saskatchewan, 2019).

Oat has many health-promoting phytochemicals, including soluble fiber, phenolic, and avenanthramides (Menon *et al.*, 2016). Its use in the human diet is becoming popular due to its ability to prevent cardiovascular disease attributed to its high β-glucan content (Anttila *et al.*, 2004). Some studies have been conducted to explore the probable genes involved in the β-glucan synthesis and regulation; however, the information has not yet explained the complete system. For instance, QTL mapping analysis was carried out and located around 250 malting-related QTLs all over the barley genome (Singh *et al.*, 2012; Wei *et al.*, 2009). QTLs on chromosome 2H, 1H, 4H, 5H, and 7H were identified to associate with β-glucan biosynthesis, thus involving to final β-glucan content (Edney *et al.*, 2004; Gao *et al.*, 2004; Han *et al.*, 1995; Hayes *et al.*, 1993; Molina-Cano *et al.*, 2007).

A similar study has been conducted in oat and found that loci *Xcdo346A*, *Xcdo82*, *Xcdo400,* and *Xcdo549B* significantly contribute to β-glucan content (De Koeyer *et al.*, 2004; Kianian *et al.*, 2000). Genome-wide association studies (GWAS) were also implemented involving numbers of different oat varieties and discovered some significant markers in β-glucan synthesis. These markers were homologous with rice *CslF*, *CslH*, and *CesA2* (Asoro *et al.*, 2013; Fogarty *et al.*, 2019; Newell *et al.*, 2012).

Subsequently, a comprehensive analysis has been conducted to identify the *Csl* and *CesA* gene families (Kaur *et al.*, 2017; Kaur *et al.*, 2016). However, these genes contribute only a part of total β-glucan content, as reported elsewhere (Newell *et al.*, 2012). Besides, given a low level of β-glucan in *Arabidopsis thaliana* CSL overexpressed transformants (Burton *et al.*, 2006;

Doblin *et al.*, 2009), it is concluded that other coding genes, enzymes, or cofactors probably involved in β-glucan synthesis and regulation (Burton *et al.*, 2006).

Recently, *Thaumatin-like protein 8* (*TLP8*) has been identified, interacting with β-glucan and regulating barley grain content (Singh *et al.*, 2017). High transcript of *TLP8* reflecting the lower β-glucan content, and vice versa (Singh *et al.*, 2017). Furthermore, the family of Thaumatin-like proteins (TLPs) in different cereals has been explored where additional germination specific TLPs were identified, which possess carbohydrate-binding motifs and have the potential for their association with β-glucan (Iqbal *et al.*, 2020). Nevertheless, no report is published about the *TLP8* function on oat β-glucan synthesis.

In this experiment, we downregulated the *TLP8* gene using the RNA interference approach in hexaploid *Avena sativa* var. park. We aimed to investigate the change of β-glucan final accumulation when *TLP8* is repressed. This technique was chosen due to its success story being applied in many organisms such as barley (Gubler *et al.*, 2008; Zalewski *et al.*, 2010), wheat (Travella *et al.*, 2006; Yan *et al.*, 2004), and rice (Ashikari *et al.*, 2005). Furthermore, the RNAi tools have been utilized successfully to overcome gene redundancy effect in higher ploidy level plants such as oat, making this technique attractive over other mutagenesis techniques (Ashikari *et al.*, 2005; Gubler *et al.*, 2008; Travella *et al.*, 2006; Yan *et al.*, 2004).

In addition, we introduced the maize *Ac* and *Ds* transposable elements into the high β-glucan diploid oat (*A. Strigosa*) to create a transposon-mediated for exploration of other genes associated with β-glucan content. It was reported that *Ac*/*Ds* transposon system had been successfully applied in many crops to improve agronomically essential traits such as yield enhancement and tolerance against drought, salinity, and cold (Jiang *et al.*, 2007). The technique has been applied in various organisms, such as soybean (*Glycine max*)(Mathieu *et al.*, 2009), *Brachypodium distachyon* (Wu *et al., 2019*), wheat (Takumi, 1996), rice (*Oryza sativa*) (Guiderdoni & Gantet, 2012; Jiang *et al.*, 2003; Shimamoto *et al.*, 1993), maize (Li *et al.*, 2013), and barley (*Hordeum vulgare*) (Ayliffe *et al.*, 2007; Koprek *et al.*, 2000; Lazarow & Lütticke, 2009). Using this system, the function of few genes in barley has been described, including the role of *WAK* genes in root elongation (Kaur *et al.*, 2013; Tripathi *et al.*, 2020), malting related genes (Singh & Singh, 2017; Singh *et al.*, 2012), and *miR172* induced spike architecture (Brown & Bregitzer, 2011; Tripathi *et al.*, 2018).

The *Ac*/*Ds* system offers valuable advantages over other techniques. It is relatively fast and straightforward where limited transformation is required (Wu *et al.*, 2019) for generating a

large number of transposon insertion lines (Wen *et al.*, 2004; Zhao *et al.*, 2001). Furthermore, the Ac/Ds system's preference to land into the linked-regions makes this approach valuable to identify the linked genes in the gene-rich region (Brown *et al.*, 2015; Koprek *et al.*, 2000; Osborne *et al.*, 1991).

To resume, In our experiment, we aimed to provide the knocked-down *TLP8* transformants and *Ac/Ds* tagging transformants, which will help a future analysis of the genes associated with β-glucan biosynthesis and regulation.

1.2 Hypotheses

In our study, we hypothesize:

1 The oat ortholog *Thaumatin-Like Protein 8* (*TLP8*) regulates β-glucan activity in *Avena sativa*

2 High β-glucan oat diploid species, *A. strigosa* is amenable to tissue culture and transformation

3 Introduction and activation of maize *Ac/Ds* elements is feasible in *A. strigosa* for the development of an efficient gene tagging approach in oat

1.3 Studies

1.3.1 Study 1: Functional Characterization of a candidate Gene (*TLP8*) Associated with β-glucan in Oat

Objectives:

1 Retrieving oat ortholog of barley *TLP8* gene (*HvTLP8*)

2 Designing of an RNAi construct

3 Development of *AsTLP8* transgenic transformants through particle gun bombardment

4 Characterization of transgenic transformants

1.3.2 Study 2: Standardizing the Tissue Culture and Transformation System and Developing Transposon Functional Genomic Resources in *Avena strigosa*

Objectives:

1 Development and standardization of tissue culture and transformation system in diploid *A. strigosa*

2 Introduction of *Ac*/*Ds* elements in diploid oat genome via particle gun bombardment

3 Generation of transgenic transformants using different *Ac*/*Ds* constructs

4 Investigation of the transposable activity of *Ac/Ds* elements

CHAPTER II: LITERATURE REVIEW

2.1 History and Importance of Oat

The archaeological evidence showed that oats had been found 32,000 years ago in the Grotta Paglicci cave in south-eastern Italy before its domestication (Lippi *et al.*, 2015). Oat was also traced in the Jordan Valley 11,500 years ago in which bulk of *Avena sterilis* (hexaploid wild oat) grains were found, demonstrated its cultivation (Kuijt & Finlayson, 2009; Sampson, 1954). Whereas, the domestication of common oat (*Avena sativa*) was recorded in the Middle of Europe 1000 BC, much later than the domestication of wheat and barley (Thomas, 1995). It is because people misunderstood oat as weeds within wheat and barley (Zohary & Hopf, 2000). After being recognized as a crop, the oat production increased significantly as favorable grains in Europe because of its unique adaptability to temperate and moist climate (Menon *et al.*, 2016).

In the seventeenth and eighteenth century, oat was a widespread cereal worldwide (Menon *et al.*, 2016). In North America, two species originally introduced were *Avena byzantina* (winter oat) and *Avena sativa* (spring oat) brought by Spanish, German, and English settlers (Menon *et al.*, 2016). By the 19th century, the area of oat cultivation was mainly centered in the east Mississippi river, followed by its cultivation in the north Mississippi valley and southern prairie Canadian province, which turned out to be the primary area of oat cultivation in North America (Menon *et al.*, 2016).

Presently, oat is a vital crop worldwide known for its phytochemicals to lower the blood cholesterol, prevent cardiovascular disease, obesity, diabetes, and coeliac disease (Anttila *et al.*, 2004; Butt *et al.*, 2008; Whitehead *et al.*, 2014). Oat is mainly used for livestock feed, human consumption, horse intake, and forage (Suttie & Reynolds, 2004). In the current years, the demand for oat for food consumption has been significantly increased due to the availability of information about its multifunctional characteristics and nutritional profile (Saskatchewan, 2019).

Globally, oat is the sixth-largest crop with a total production of 22.51 million metric tons (Figure 1) (USDA, 2019). Russia is the largest oat producing country, covering about 30% of total global production (Flynn & Smith, 2010). Canada and Australia follow it as the second and third-biggest producers, with the whole production are about 2.9 and 1.5 million metric tons, respectively (Flynn & Smith, 2010; USDA, 2019).

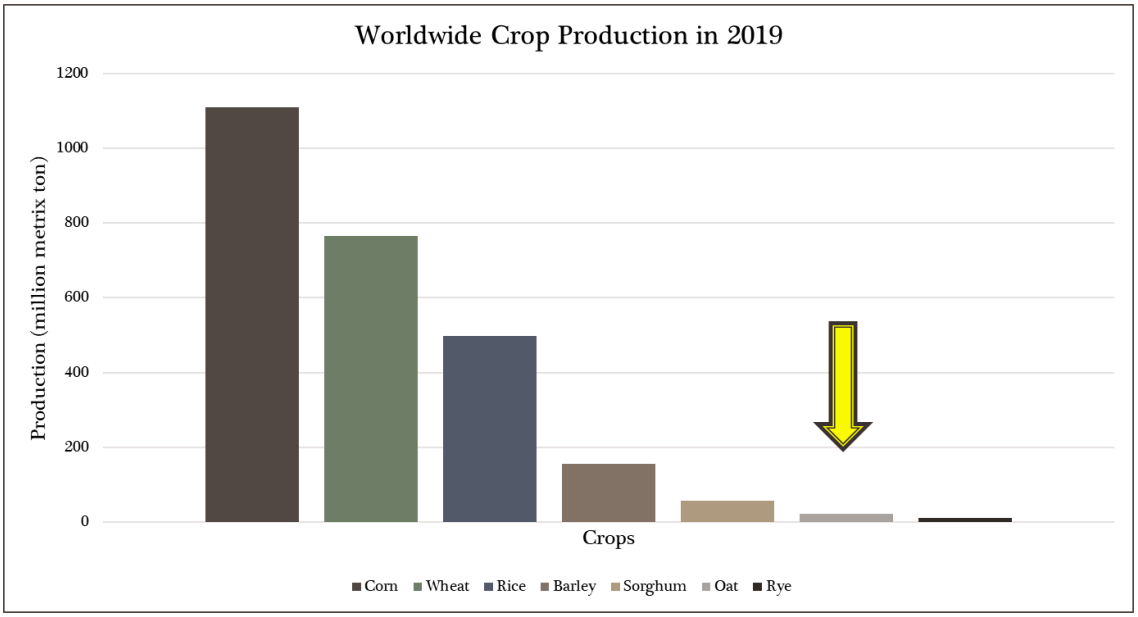

Figure 1. The worldwide crop production of cereal crops in 2019. Oat ranked sixth with the production of 22.51 million metric tons.

In the worldwide oat trade (Figure 2), the total production and consumption have increased by years, especially in 2016-2018 (USDA, 2019). Although the production went down slightly in 2018/2019, the positive signal was shown by the end of 2019, with the total production and consumption has reached about 22,513 and 22,270 thousand metric tons (USDA, 2019). The export data displays the same trend as production and consumption. The highest export rate was reached in 2017/2018 (POGA, 2020; USDA, 2019). At the end of 2019, an increase started after the significant fall-off (POGA, 2020; USDA, 2019).

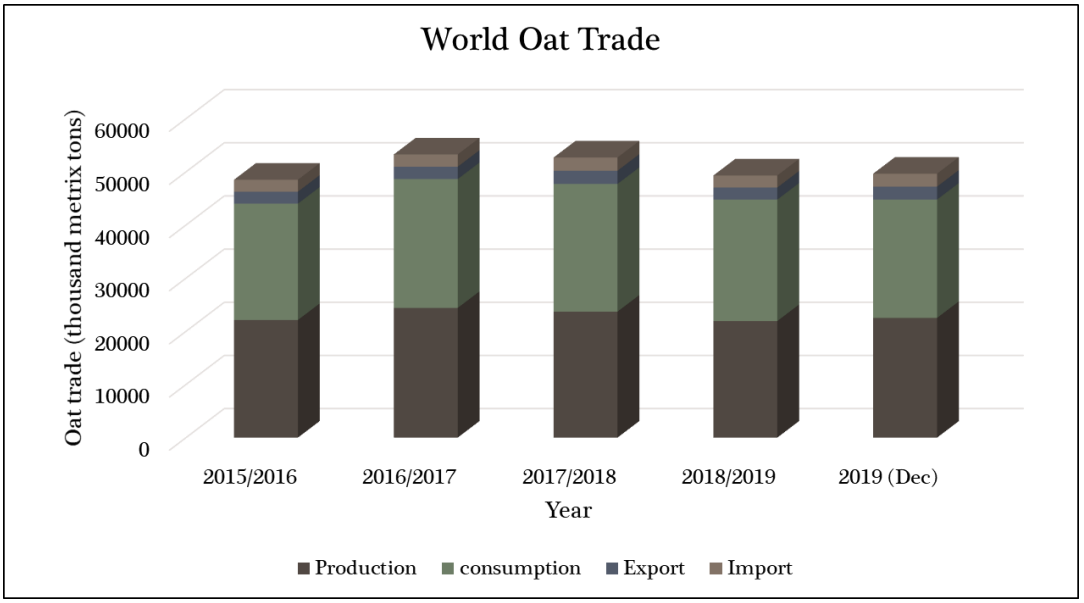

Figure 2. The world oat trade, including production, consumption, export, and import rate from 2015-2019.

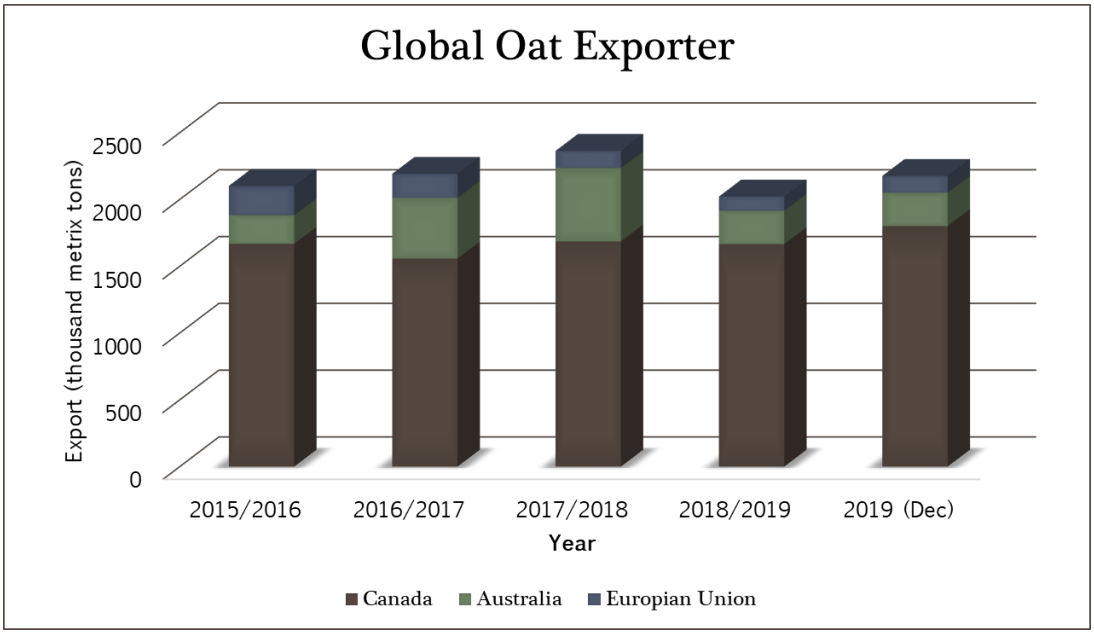

Figure 3. The worldwide oat exports from 2015-2019. The graph shows the three largest oat exporters globally (Canada, Australia, and European Union).

Canada is one of the largest oat exporters with a 60% share globally (Figure 3), making Canada a critical country for the worldwide oat stock (POGA, 2020; USDA, 2019). Canadian oats are mainly exported to the USA and Mexico (POGA, 2020). Moreover, Canada is currently expanding its market through the Trans-Pacific Partnership (CPTTP) countries (POGA, 2020).

2.2 Oat Taxonomy and Genome

Oats belong to the genus *Avena* under the Poaceae family, with three ploidy levels; diploid, tetraploid, and hexaploid (Ladizinsky & Zohary, 1971). Using different criteria, researchers have identified 7-30 distinguished oats species (Baum, 1977; Ladizinsky & Zohary, 1971). The first classification was done by Linnaeus in 1753 and 1762 (Linnaeus, 1762) followed by other taxonomists. The most recent *Avena* taxonomy was published in 2008 by Loskutov, who divided the *Avena* genus into 26 species based on their differential morphology, the karyotype structure, RAPD confirmation, and the Avenin spectrum analysis (Figure 4) (Kole, 2011; Loskutov, 2008).

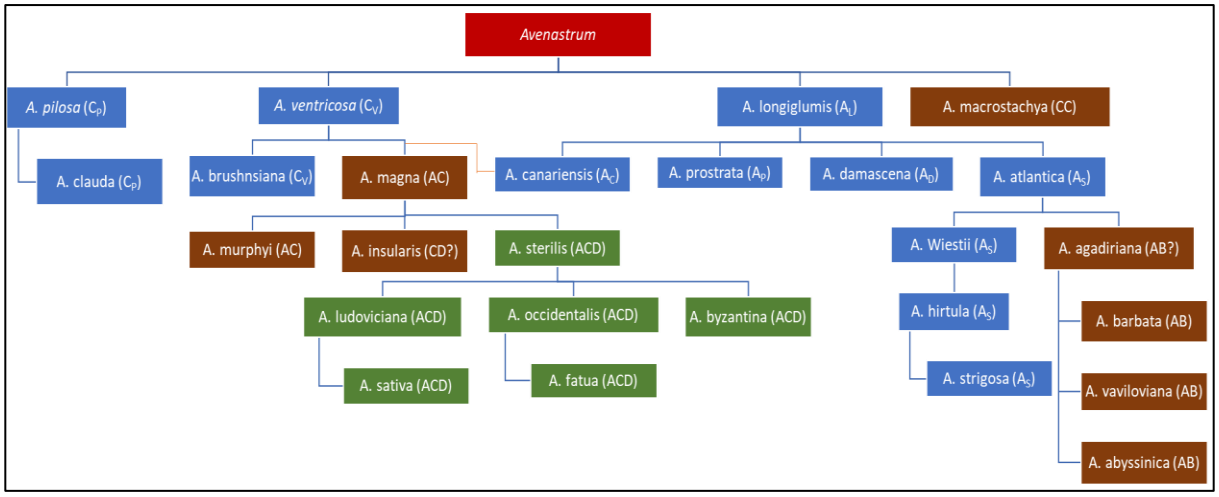

Figure 4. Diagram of Phylogenetic relationship in Avena genus under subgenus Avenastrum (Loskutov, 2008)

Oat has x=7 basic chromosome number, in which the diploid oat contains A and C genome, tetraploid oat has AC and AB genome, and hexaploid oat has ACD genome (Singh & Upadhyaya, 2015). The ancestor of either B or D genome has not been confirmed yet; however, most of the species resemble the A genome (Singh & Upadhyaya, 2015). Diploid A and C genomes share many genome features; however, the small variations are observed within the same genome (Thomas, 1995). These differences subsequently created the genome variants, including Al (*A. longiglumis*), Ad (*A. damascene*), Ac (*A. canariensis*), Ap (*A. prostrata*), As (*A. atlantica, A. wiestii, A. hirtula, A. strigosa*) for A diploid genome and Cp (*A. Pilosa, A. clauda*), Cv (*A. ventricosa, A. bruhnsiana*) for diploid C genome (Figure 4)(Thomas, 1995).

One of the distinguishing factors of the A and C genome is observed from the karyotype structure and plant morphology (Leggett & Thomas, 1995). Diploid A genome confers a symmetrical karyotype, a chromosome that is more primitive than asymmetrical type, possessed by the C diploid genome (Leggett & Thomas, 1995). However, the plant with the C genome has more likely resembled archaic oat morphology that experienced a slower rate of evolution (Stebbins, 1971).

The tetraploid oat has two different forms, AC and AB genome (Ladizinsky & Zohary, 1971). As the B genome's ancestral donor is not known, it is proposed that the AB tetraploid oat has highly likely derived from the As diploid genome variant type (Ladizinsky & Zohary, 1971). The AB polyploid is formed through autopolyploidization, in which two sets of the chromosome are derived from the same parental species, for instance, *A. abyssinica*, *A, vavilovianaa*, *A.*

barbata, and *A. agadiriana* (Figure 4) (Ladizinsky & Zohary, 1971). It differed from AC genome polyploidization, which is originated from different parental species, A and C diploid genomes (Ladizinsky & Zohary, 1971). This genome group constitutes *A. murphyi* and *A. magna* species (Figure 4) (Ladizinsky & Johnson, 1972).

The hexaploid ACD oat genome is originated from AC tetraploid oat genome through the hybridization of the diploid A and C genomes, followed by doubling the chromosome number (Chew *et al.*, 2016; Fu, 2018; Li *et al.*, 2000; Loskutov, 2008; Thomas, 1992). It includes *A. fatua*, *A. occidentalis*, *A. byzantina*, *A. sativa*, *A. ludoviciana*, and *A. sterilis* (Figure 4) (Loskutov, 2008). It is concluded that the oat genome evolution is mainly originated from the A and C diploid genomes (Loskutov, 2008). While other forms are likely the variants of these forms (Loskutov, 2008).

The genome size is different, mainly due to the ploidy level, degree of transposon amplification, and evolutionary history (Bennetzen *et al.*, 2005). Species with a higher ploidy number predominantly have a larger genome size than the species with lower ploidy levels (Gerstein & Otto, 2009). Therefore, the different oat ploidy levels resulted in various genome sizes (Yan *et al.*, 2016). Furthermore, Yan *et al.* (2016) reported that the genome type influenced its size, in which C genome type are more extensive than A genome. Therefore, tetraploid CC plants are comparatively larger than AC and AB plants (approximately 2C= 21.78 pg, 18.60 pg, and 16.76 pg, respectively) (Brown *et al.*, 2015). In addition, observation has been made that the hexaploid ACD genome size (about 2C= 25.74 pg) has not been much different than the diploid CC plants (2C= 21.78) (Yan *et al.*, 2016).

2.3 Nutritional content of Oat

Oat is mainly used as animal feed; however, its importance has been increased recently for human consumption due to its health-promoting phytochemicals (Singh & Upadhyaya, 2015). Among different grain crops, oat has the highest protein content ranging between 12.4-24.4% (Pomeranz *et al.*, 1971). In general, protein is mainly found in the germ and bran but less prevalent in the endosperm (Singh & Upadhyaya, 2015). Some species that contain considerably high protein (over 20%) are diploid *A. atlantica, A. canariensis, A. clauda, A. damascena, A. hirtula, A. longiglumis, A. pilosa, A. strigosa, A. ventricose*, tetraploids *A. agadiriana, A. barbata, A. magna, and A. Murphyi*, and hexaploid *A. fatua* and *A. sterilis, A. ludoviciana, A. occidentalis* (Singh & Upadhyaya, 2015).

Oat protein composition is considerably different from other cereals. Generally, wheat, barley, and rye have a high percentage of prolamin, an alcohol-soluble protein component (Klose & Arendt, 2012). Nevertheless, oat constitutes of high salt-water soluble globulins than prolamins (Klose & Arendt, 2012). Oat has also been considered nutritionally rich because of essential amino acids like lysine and threonine (Klose & Arendt, 2012). Furthermore, oat contains some antioxidants such as avenanthramide, phytic acid, and ferulic acid that are essential for human health (Kemppainen *et al.*, 2010), as well as many essential vitamins and minerals such as vitamin B1, zinc, manganese, iron, phosphorus, and copper (Welch, 1995).

The high content of dietary fiber, especially the 1,3;1,4-β-D-glucans (β-glucan) in oat, has been reported to lower cholesterol and blood pressure (Braaten *et al.*, 1994). Furthermore, regulatory authorities confirmed that consuming oat 3 grams per day would reduce heart disease risk (Anderson *et al.*, 1984). Studies indicate that β-glucan regulates blood sugar, helping people who have type II diabetes (Lammert *et al.*, 2008). Experiments conducted with type II diabetes patients showed a substantial reduction in insulin dosage (40%) after consuming oatmeal for 4-weeks (Lammert *et al.*, 2008).

A high amount of β-glucan in oat probably increases the satiety feeling as it will stay longer in the stomach, yield the satiety hormone, and intensify the stomach distension (Tosh, 2013). Oat is low in calories and high in fiber makes oat an excellent diet for weight loss (Tosh, 2013). Additionally, oat has a negligible level of gluten protein that is the best diet for people who have celiac disease (Kemppainen *et al.*, 2010). Furthermore, oat's anti-inflammatory properties have proven clinically to heal itchy and dry skin (Kemppainen *et al.*, 2010).

2.4 1,3;1,4-β-D-glucan (β-glucan) components in oat

The β-glucans are the major components of dietary fiber that are highly beneficial in preventing cardiovascular disease, high serum cholesterol, obesity, and non-insulin-dependent diabetes (Anttila *et al.*, 2004). Significantly, β-glucans form a viscous layer in the gut (Anttila *et al.*, 2004). Its capability to hold water and bind to the bile acid reduces glucose and cholesterol absorption in the blood (Anttila *et al.*, 2004). Higher viscosity in the small intestine leads to more extended breaking downtime of glucose after a meal (Hodge *et al.*, 2004). Thus, it reduced the glycemic index and benefited people with type II diabetes (Hodge *et al.*, 2004). Moreover, in the large intestine, β-glucans are fermented into small chain fatty acids that could reduce colon cancer risk (Ferguson, 2003).

In 1997, the FDA announced that consuming 3 grams per day of β-glucans could decrease the saturated fat and heart disease risk (Health & Services, 1997). It is the first time the government claimed that dietary fiber consumption could prevent diseases (Health & Services, 1997). Since then, the attraction to oat β-glucans and its research increased substantially (Health & Services, 1997).

Furthermore, β-glucans are well documented as having immunostimulant properties (Akramienė *et al.*, 2007). They enhance the immune activity either innate or specific body immune system by activating particular receptors on macrophage, neutrophils, and natural killer (NK) cells or indirectly activating pinocytic M-cells located in the prayer's patches in the small intestine (Akramienė *et al.*, 2007; Bohn & BeMiller, 1995). It is accomplished by increasing the pro-inflammatory cytokine production, chemokine production, and oxidative burst (Akramienė *et al.*, 2007). β-glucan also acts as an antitumor agent and provides the ability to resist pathogen attacks such as viruses, bacteria, protozoa, and fungi (Akramienė *et al.*, 2007; Mantovani *et al.*, 2008).

β-glucan is a unique feature of the cell-wall of the *Poaceae* family grains (Smith & Harris, 1999). Nonetheless, this polysaccharide can be found in other related families and taxa but with a minimal amount (Harris & Fincher, 2009). The content of β-glucan varies among species as identified in oat 3-8g/100g dry weight (DW), barley 2-20g/100g (DW) (Harris & Fincher, 2009), sorghum 1.1–6.2g/100g (DW) (Ogbonna & Egunwu, 1994), rye 1.3–2.7g/100g (DW), maize 0.8–1.7g/100g (DW) (Fincher & Stone, 2004), triticale 0.3–1.2 g/100g (DW) (Bacic *et al.*, 2009), wheat 0.5–1.0 g/100g (DW), durum wheat 0.5-0.6 g/100g (DW) (Beresford & Stone, 1983), and rice 0.13g/100g (DW) (Anderson *et al.*, 1978). It is also reported that β-glucans vary in different environmental conditions during endosperm development (Harris & Fincher, 2009).

The β-glucan constitutes an unbranched and unsubstituted chain of glucosyl residues through the 1.3 and 1.4 linkages (Harris & Fincher, 2009). The β-glucans complex predominantly consists of units composed of two or three 1.4 linkages separated by 1.3 linkages, namely cellotriosyl and cellotetraosyl units (Harris & Fincher, 2009). Overall, cellotriosyl and cellotetraosyl composed about 90% of total cereal polysaccharides, with the longer chains remaining 10% (Lazaridou & Biliaderis, 2007). Different ratios of cellotriosyl and cellotetraosyl in wheat (3.0-4.5: 1), Rye (1.9-3.0: 1), barley (1.8-3.5: 1), and oat (1.5-2.3: 1) affect the solubility of β-glucans in water (Lazaridou & Biliaderis, 2007). Wheat possessing a relatively

high cellotriosyl and cellotetraosyl ratio of β-glucan is more insoluble in water than oat (Li *et al.*, 2006). Similarly, barley with more cellotriosyl and cellotetraosyl contains less soluble fraction (65%) than oat (82%) (Li *et al.*, 2006).

Genetic and environmental components influence the content of oat β-glucans (Welch, 1995). Overall, the diploid oat with A genome contains relatively high β-glucans compared to tetraploid and hexaploid oat. The highest β-glucan content has been found in *Avena atlantica* with a total of 11.3% (Table 1) (Welch *et al.,* 2000).

Table 1. The total β-glucans content in different oat genotypes (Redaelli et al., 2013; Welch et al., 2000)

Species	Genome	Total β-glucans (%)
Diploid oat, 2n= 14		
Avena canariensis	Ac	3.57
Avena damascena	Ad	6.77
Avena hirtula	As	4.99-6.28
Avena strigosa	As	2.85-6.23
Avena wiestii	As	6.55
Avena atlantica	As	5.7-11.3
Avena damascene	Ad	4.8-7.9
Avena longgligumis	Al	3.1-3.8
Tetraploid oat, 2n= 28		
Avena abyssinica	AB	3.58-5.12
Avena barbata	AB	4.51-5.46
Avena agadiriana	AB	3.00-3.30
Avena maroccana	AC	2.40-3.30
Avena murphy	AC	4.2-5.4
Hexaploid oat, 2n= 42		
Avena byzantine	ACD	2.88-4.94
Avena fatua	ACD	3.20-5.90
Avena hybrida	ACD	4.23
Avena sativa	ACD	3.02-5.74
Avena sterilis	ACD	3.90-5.22
Avena occidentalis	ACD	4.30-5.80

2.5 Genetic of β-glucan

The role of β-glucan is well documented for its association with many health attributes; however, relatively little is known about its biosynthesis and regulation. The first identification of genes involved in β-glucans biosynthesis was conducted by the quantitative trait loci (QTL) mapping analysis followed by identifying candidate genes in the regions associated with β-glucan (Singh et al., 2017).

Many research groups have conducted QTLs analyses to determine the possible genomic regions involved in the β-glucan biosynthesis and regulation. Around 250 malting-related

QTLs have been located all over the barley genome (Wei *et al.*, 2009). Han *et al.* (1995) discovered that QTLs on chromosome 2H and 1H were identified to have the most considerable effect on β-glucan. Another major QTL, QTL2, contributes 28.9% to 37.6% variation for malt β-glucan and malt extract percentage, respectively (Han *et al.*, 1997). The QTL2 has been located on the telomeric region of the short arm of chromosomes 4H, around 15.8 cM (Gao *et al.*, 2004; Hayes *et al.*, 1993).

In addition, Edney *et al.* (2004) investigated doubled haploid lines from an Arapiles/Franklin cross. They identified the significant QTL for β-glucan regulation during the malting process on chromosome 4H, which coincidently is located with the *heat-stable beta-amylase* (*sd2H*) gene. Furthermore, Molina-Cano *et al.* (2007) has characterized three QTLs associated with β-glucan from the study of a cross between European and North American two rows malting barleys. One QTL was located on chromosome 1H, shared the same region of a gene involved in the synthesis of cell wall polysaccharides (Molina-Cano *et al.*, 2007). The other two QTLs were found on chromosome 5H and 7H, the same location of the genes regulated grain protein content and hulless grain, respectively (Molina-Cano *et al.*, 2007).

Whereas, several genetics studies have also been conducted to identify oat β-glucan synthesis and regulation. Kianian *et al.* (2000) used single-factor ANOVA, simple interval mapping (SIM), and simplified composite interval mapping (sCIM) to identify the quantitative trait loci (QTLs) in two recombinant inbred lines (Kanota x Ogla (KO) and Kanota x Marion (KM)). In the KO population, it was discovered that linkage group KO3, KO6, KO11, KO13, KO14, KO17, and KO20 significantly contribute to β-glucan content, with the linkage group KO3 has the most considerable effect on β-glucan content, which contributes to 3.5 g/kg of β-glucan (Kianian *et al.*, 2000). The loci in group 3 included *Xcdo346A*, *Xcdo82*, and *Xcdo549B* (Kianian *et al.*, 2000). Furthermore, in the KM population, four genomic regions were discovered, significantly affecting the β-glucan content. These regions include linkage groups KM11, KM14, KM5X, and KM10X, with the most important locus has been found on the linkage group KM14, known as *Xcdo400* (Kianian *et al.*, 2000). This locus contributed to a change of 3.7 g/kg in β-glucan content (Kianian *et al.*, 2000). Besides, using AFLP, RFLP, RAPD, SCAR, and phenotypic marker, De Koeyer *et al.* (2004) evaluated a population of two Canadian spring oat varieties Terra and Marion (TM) and discovered that TM5, TM18, TM21, and TM29 linkage group significantly affected β-glucan content.

Genome-wide association studies (GWAS) were also used as an alternative approach for QTL detection. Newell *et al.* (2012) have implemented the study to identify the loci affecting β-glucan content in 431 diverse lines using the Diversity Array Technology (DArT) marker. Three independent markers were discovered and significantly associated with β-glucan content (Newell *et al.*, 2012). These markers were aligned with the rice genome and revealed that one was homologous with the rice *Cellulose Synthase-Like F* (*CslF)* gene family located on chromosome 7 (Newell *et al.*, 2012). A similar study was conducted by Asoro *et al.* (2013) involving 446 North American Oat elite lines. Twenty-four markers were associated with β-glucan. Two of them were adjacent to C*ellulose Synthase A Catalytic Subunit 2* (*CesA2)* gene, another candidate gene involved in the β-glucan biosynthesis (Asoro *et al.*, 2013).

With the development of a new 6K single-nucleotide polymorphism (SNP) and GBS-based SNPs, Fogarty *et al.* (2019)(2019) performed an updated GWAS using three elite accessions (Spring, Winter, and World Diversity) grown in multiple locations in North America. Fifty-eight significant markers were discovered, with 4 of them were homologous with barley *CslF* and *CslH* gene families along with *UDP-Glucose Pyrophosphorylase* (*UGPase*) and *ADP-Glucose Pyrophosphorylase* (*AGPase*) candidate genes (Fogarty *et al.*, 2019). Also, four overlapping QTLs included one from Mrg05, two from Mrg06, and one from Mrg11, were mapped on barley chromosome 2, where *Cellulose Synthase A1* (*CESA1*) is located (Fogarty *et al.*, 2019). Comprehensive analysis of *CESA* and *CSL* families in small grain cereals has been recently reported (Kaur *et al.*, 2017; Kaur *et al.*, 2016).

The subsequent confirmation was conducted by overexpression of the *CslF* gene in *Arabidopsis thaliana,* which contains no β-glucan (Burton *et al.*, 2006; Doblin *et al.*, 2009). Using immunocytochemical and enzymatic methods, *CslF* expression was detected in the cell wall (Burton *et al.*, 2006). A similar analysis was conducted to explore another gene from the same family of *CslF* and found that the *CslH* gene also contributes to the synthesis of β-glucan (Doblin *et al.*, 2009). Therefore, both *CslF* and *CslH* genes are required to synthesize β-glucan (Burton *et al.*, 2006; Marcotuli *et al.*, 2016; Nemeth *et al.*, 2010). Both genes belong to *Cellulose Synthesis-Like families*, which confer D, D, TED, and QxxRW motifs with the active binding site and nucleotide sugar-binding site (Richmond & Somerville, 2000). However, the overexpression of CslF and CslH genes in Arabidopsis thaliana synthesizes only a small amount of β-glucan (Marcotuli *et al.*, 2016).

Recently, Singh *et al.* (2017) dissected an important barley QTL2 on chromosome 4H associated with β-glucan and malt extract using expression and microsynteny-based approach. Analysis of the QTL2 region identified that Thaumatin-like protein 8 (TLP8) interacts with β-glucan and regulates its content in the germinating grain. Higher transcript abundance of *TLP8* in barley grains reflects lower amounts of β-glucan, and vice-versa (Singh et al., 2017). Furthermore, the family of Thaumatin-like proteins (TLPs) in different cereals has been explored where additional germination specific TLPs were identified, which possess carbohydrate-binding motifs and have the potential for their association with β-glucan (Iqbal *et al.*, 2020).

2.6 Genomic Approaches to Understand gene function

2.6.1 RNA interference

RNA interference (RNAi) is the mechanism induced by small double-stranded RNA (20 – 24 bp) involving some key enzymatic features such as Argonaute (AGO) and Dicer or Dicer-Like (DCL) proteins to downregulate the gene expression (Meister & Tuschl, 2004). The first RNAi work was published in 1928 (Baulcombe, 2004). The mechanism is started by the cleavage of long double-stranded RNA (dsRNA) by DCL to form 20-24 bp small RNA, which is then processed by AGO protein to generate single-stranded RNA (sRNA) guide strand (Guo *et al.*, 2016; Meister & Tuschl, 2004). This, along with other proteins, generates an RNA-induced silencing complex (RISC) (Meister & Tuschl, 2004). The system scrutinizes the genome to recognize the specific messenger RNA (mRNA) that share the complementary sequence with the guide strand RNA to induce the mRNA cleavage and degradation (Guo *et al.*, 2016; Meister & Tuschl, 2004).

The diversity of RNA silencing pathways

Plants have different silencing pathways according to the source of dsRNA and the functional target. **The first pathway is endogenous silencing mRNA by micro RNA (miRNA).** The miRNA is a 21-24 bp long nucleotide resulted from the Dicer cleavage of precursor miRNA (Baulcombe, 2004). Plant *microRNA* (*MIR*) genes are non-coding sequences played as the primary miRNA source (Baulcombe, 2004; Guo *et al.*, 2016). These genes were transcribed into primary microRNA by RNA polymerase II (Baulcombe, 2004). The Pri-mRNA forms a hairpin structure due to the presence of the complementary sequence (Baulcombe, 2004). The structure is shortened by DCL protein, primarily DCL1, and processed into precursor

mRNA (pre-mRNA) in the nucleus (Baulcombe, 2004). DCL1 further processes the pre-mRNA to form the miRNA duplex consisting of a guide strand (mature mRNA) and passenger strand (Baulcombe, 2004). The duplex is methylated at 3' terminal region by *Hua Enhancer1* (*HEN*) to protect the structure from degradation while being exported into the cytoplasm (Baulcombe, 2004). Ultimately, the guide strand RNA will incorporate AGO protein to form an RNA-induced silencing complex (RISC) (Guo *et al.*, 2016; Vazquez *et al.*, 2008). Primarily the miRNA targets the 3' untranslated region (UTR) messenger RNA, which functions to induce mRNA degradation or translation repression (O'Brien *et al.*, 2018). However, it also has been reported that miRNA could target the 5' UTR, coding gene, and promoter region (O'Brien *et al.*, 2018).

The second pathway is the RNA-directed DNA Methylation (RdDM). RdDM Pathway is de novo cytosine methylation, which is induced by 24-nt siRNAs that are generated by a successive process of Polymerase IV (Pol IV), RNA DEPENDENT RNA POLYMERASE II (RDRII), and DICER-LIKE 3 (DCL 3) (Matzke *et al.*, 2015). In the canonical pathway, the siRNAs then recruit AGO4 to form the siRNA-AGO4 complex (Matzke *et al.*, 2015). Finally, the form attracts the DOMAIN REARRANGED METHYLTRANSFERASE II (DRM II) to de novo methylate the RNA-DNA sequence homology assisted by scaffold RNA generated by plant-specific RNA Polymerase V (Pol V) (Matzke *et al.*, 2015). The RdDM pathway mainly targets transposon and repetitive genomic sequence (Corem *et al.*, 2018; Matzke *et al.*, 2015). De novo cytosine methylation at the symmetrical CH and CHG region (where H is any nucleotide but G) are maintained over the generation by Methyltransferase1 (MET1) and Chromomethylase3 (CRM3), respectively (Matzke *et al.*, 2015). However, the CHH methylation site cannot be preserved over a generation; therefore, it entirely depends on the RdDM pathway (Matzke *et al.*, 2015). The recent studies have found the alternative RdDM pathway, which requires Pol II, RDR 6, DCL2, and DCL4 to generate 21 and 22-nt siRNA (McCue *et al.*, 2015). The siRNA processed with AGO4 and AGO6 to bind the PolV-dependent RNA scaffold and recruit AGO1 to cleave the transcript as the PTGS process (Matzke *et al.*, 2015; McCue *et al.*, 2015)

The third pathway is the RNA silencing induced by an exogenous nucleic acid. RNA silencing could be induced by exogenous nucleic acid through the virus attack or sense transgene transmission, typically consisting of long base-paired dsRNA (O'Brien *et al.*, 2018). The dsRNA is processed by Dicer to form siRNA to function silencing (Carthew & Sontheimer, 2009; O'Brien *et al.*, 2018). Formerly, it is a natural defense against the virus (Carthew & Sontheimer,

2009; O'Brien *et al.*, 2018). Furthermore, it has been found that the endogenous repetitive region, centromere, and transposon are also the precursor for endogenous siRNA (Lippman & Martienssen, 2004).

Sense transgene could be silenced through Transcriptional Gene Silencing (TGS) and or Post Transcriptional Gene Silencing (PTGS). The mechanism is induced by multiple transgene copies. These copies are recognized by key enzymatic proteins such as RDR2 and PolIV to produce 24-nt siRNA, leading to methylation through the RdDM pathway and mediating the transcriptional silencing (Waterhouse *et al.*, 2001). Furthermore, multiple repeats single-stranded RNA transgene induces the dsRNA and long hpRNA processed by DCL protein and leads to full PTGS (Waterhouse *et al.*, 1998; Waterhouse *et al.*, 2001).

RNA sources from viral infection could be either the inducer or target of the RNA silencing pathway, which is considered as the anti-viral defense system in plants (Moissiard & Voinnet, 2006). The dsRNA is processed through the host plant siRNA pathways that involve DCL4 and DCL2 protein to generate 21-nt and 22-nt siRNA (Moissiard & Voinnet, 2006). The siRNA then recruits AGO protein to target the viral RNA itself (Guo *et al.*, 2016). For DNA viruses, both replication and transcription occur in the plant host nucleus and require DCL3 to generate 24-nt siRNA (Yadav & Chattopadhyay, 2011). The viral DNA is targeted at the transcriptional level through the methylation in the promoter region (Yadav & Chattopadhyay, 2011). While the viral derived transcripts are targeted at the post-transcriptional level (Yadav & Chattopadhyay, 2011).

Based on the fundamental knowledge of dsRNA induced silencing pathway in plants, artificial silencing technology has been developed. It is mainly aimed at understanding the gene function via a reverse genetic approach and developing a protection system against viral invasion. One of the techniques developed is hairpin RNA (hpRNA) transgene. The constructs have been designed by joining sense and antisense perfect complementary sequence in an inverted orientation (Smith *et al.*, 2000). The sequences are separated by intron intended to stabilize the structure and increase the RNA silencing efficiency up to 100% when targeted against viruses and endogenous genes (Smith *et al.*, 2000). The sense and antisense transcript RNA complement each other and form dsRNA (Wesley *et al.*, 2001). The dsRNA then activates the silencing machinery started by the cleavage of long dsRNA by generating 21, 22, and 24-nt siRNA processed by DCL4, DCL2, and DCL3, respectively (Guo *et al.*, 2016). The sense siRNA

integrated with AGO and other proteins generate RISC to target mature mRNA silencing (Guo *et al.*, 2016).

2.6.2 RNAi as Functional Genomic Resource

RNAi as a functional genomic tool has been applied in various plants, for example, in Arabidopsis (Kerschen *et al.*, 2004), potato (Eschen-Lippold *et al.*, 2012; Schwind *et al.*, 2009), papaya (Kertbundit *et al.*, 2007), tobacco (Waterhouse *et al.*, 2001), wheat (Travella *et al.*, 2006; Yan *et al.*, 2004), barley (Gubler *et al.*, 2008; Zalewski *et al.*, 2010), rice (Ashikari *et al.*, 2005). Mainly, RNAi is applied to downregulate specific genes for establishing the function of genes through mutant analysis.

RNAi has been used to improve agriculturally important traits, such as accelerating flowering by knocking down of *VRN2* gene (Yan *et al.*, 2004) and *Terminal flower 1* (*TFL1*) gene (Freiman *et al.*, 2012), enhancing grain yield by down regulating *OsCKX2* gene (Ashikari *et al.*, 2005), increasing dormancy by silencing of *HvABA8'OH1 gene (Gubler et al., 2008),* delaying leaf senescence by silencing *Carotenoid Cleavage Dioxygenase* (*CCD*) genes (Ledger *et al.*, 2010), and extending shelf-life in vegetables and fruits by down regulating of *1-Aminocyclopropane-1-Carboxylate Oxidase* (*ACCO*) gene (Xiong *et al.*, 2005). Also, RNAi has been used to improve the performance of plants. Several studies have been carried out to identify and understand the plant architecture at the molecular level. For example, the reduction of *Carotenoid Cleavage Dioxygenase* (*CCD*) genes that are believed to play an essential role in branch development resulted in increased branch number (Ledger *et al.*, 2010). Silencing mechanism targeted against *Auxin Response Factor 7* (*ARF7*) and *Chalcone Synthase* (*CHS*) in tomato has resulted in seedless fruits (De Jong *et al.*, 2009; Schijlen *et al.*, 2007).

Other approaches, such as altering the flower color and scent, have also been applied to meet the market value and consumer needs. For instance, targeting *Chalcone Isomerase* (*CHI*) (Nishihara *et al.*, 2005), *Anthocyanin 5,3'-Aromatic Acyltransferase* (*5/3' AT*), *Flavonoid 3', 5'-Hydroxylase* (*F3'5'H*) (Nishihara & Nakatsuka, 2011), and *Flavonoid 3-O-Glucosyltransferase* (*UFGT*) (Chen *et al.*, 2011) genes via silencing mechanism has successfully resulted in the color change to display various color expressions. Furthermore, the flower's scent has also been modulated through RNAi silencing. For example, by downregulating the genes, including *Phenyl-ethanol Benzoyl-Transferase* (*PhBSMT*), *Benzyl-alcohol* (*PhBPBT*), and *Coniferyl Alcohol acyl-Transferase* (*CFAT*) (Dexter *et al.*, 2007; Kaminaga *et al.*, 2006). All these silencing efforts could help to improve plant productivity as well as their market value.

RNAi offers many advantages over other techniques. It is a powerful technique that could be applied to overcome gene redundancy problems, especially in higher ploidy crop species, such as wheat and oat (Ashikari *et al.*, 2005; Gubler *et al.*, 2008; Travella *et al.*, 2006; Yan *et al.*, 2004). Moreover, RNAi has advantages over antisense mediated approach in terms of its efficiency and stability (Kusaba, 2004).

Recently, an advanced tool has been developed, known as Clustered Regularly Interspaced Short Palindromic Repeats and associated endonuclease (CRISPR-Cas system). However, RNAi offers advantages over CRISPR-Cas gene editing, including 1) the complete knock-out might lead to lethality; therefore, such mutants could not be recovered by CRISPR-Cas (Guo *et al.*, 2016). However, such mutants could be functionally recovered by RNAi silencing. 2) The RNAi mechanism allows the silencing in the targeted tissue or organ by applying a specific promoter (Liu *et al.*, 2020). However, the knock out by CRISPR would target all tissues and organs. 3) The RNAi technology could overcome the gene redundancy challenges in different ploidy level plants, which is difficult to achieve using CRISPR (Guo *et al.*, 2016).

2.7 Plant Transposable Elements

Transposable elements (TE) are the genomic sequences that can transpose from their original position and reinsert into a new chromosomal region (McClintock, 1951). TEs were first discovered by Barbara McClintock, while examining the variegation in maize kernel and chromosome breakage at specific sites (McClintock, 1951). She later compared and investigated their correlation and found that the insertion of a specific sequence caused the changes into the chromosome, which was named “controlling elements” (McClintock, 1951). This discovery led her to receive the Nobel award in 1983 in the physiology and medicine field (The Noble Prize, Nobel Media AB 2020). The first transposable elements characterized by McClintock were *Ds* (*Dissociation*) and *Ac* (*Activator*) that cause the break at maize chromosome number 9 (The Noble Prize, Nobel Media AB 2020). With the advances in sequencing technologies, TEs have been identified as the most extensive genetic component of most eucaryotes. Approximately 45% of the human genome (Lander et al., 2001) and up to 70-80% genome of grass species such as maize, wheat, and oat (Sabot *et al.*, 2004) are comprised of TEs.

According to different intermediate used during transposition, TEs are divided into two main classes (Feschotte *et al.*, 2002). The TEs class I composed of RNA as intermediate, while TEs class II uses DNA as intermediate (Feschotte *et al.*, 2002). Both groups have autonomous and

non-autonomous elements (Du *et al.*, 2011). The autonomous class consists of open reading frames that encode the transposase enzyme required for transposition activity (Du *et al.*, 2011). While in the non-autonomous group, transposase genes are either lacking or mutated in another form having only the cis-recognition site to promote TE transcription and facilitate transposition (Feschotte *et al.*, 2002).

Transposable Elements (TEs) Class I: Retrotransposon

TEs Class I have RNA as an intermediate in the transposition event. This class is generally known as RNA transposons or as Retrotransposon (Sabot *et al.*, 2004; Wicker *et al.*, 2007). The RNA is reverse-transcribed into DNA by reverse transcriptase either indirectly generated in the cytoplasm as VLP (virus-like particle) or directly in the new location in the genome, followed by integration via integrase and copy-paste mechanism (Sabot *et al.*, 2004; Wicker *et al.*, 2007). The different reverse-transcribed DNA production and structure distinguish two sub-classes of class I TEs (Sabot *et al.*, 2004; Wicker *et al.*, 2007).

Subclass I

Subclass I of retrotransposon produces DNA through a cytoplasmic reverse-transcribed DNA intermediate (Sabot *et al.*, 2004; Wicker *et al.*, 2007). It constitutes a long terminal repeat of various sizes ranging between 100bp to 5kbp and is termed Long Terminal Repeat (LTR)-Retrotransposon (Feschotte *et al.*, 2002). The LTR region comprises three elements; U3: Unique 3' RNA, R: Repeat RNA, U5: Unique 5' RNA (Feschotte *et al.*, 2002). RNA polymerase II promoter and transcription initiation site are located within this area (Feschotte *et al.*, 2002). In an autonomous retrotransposon, there are at least two gag proteins, a structural protein for virus-like particles, and Pol protein, which is comprised of three or more open reading frames within LTR, including reverse-transcriptase (RT), protease (PR), RNAseH, and integrase (INT) (Feschotte *et al.*, 2002; Sabot *et al.*, 2004; Wicker *et al.*, 2007). The non-autonomous class lacks almost all these coding sequences (Feschotte *et al.*, 2002; Sabot *et al.*, 2004; Wicker *et al.*, 2007).

Classification of subclass I retrotransposons is shown in Table 2, which includes the following major groups. **1. *Copia* group**. The pol sequence structure is formed of PR-INT-RT-RNAseH, which is 8 kb long (Figure 5) (Sabot *et al.*, 2004; Wicker *et al.*, 2007). **2. *Gypsy* group**. The pol sequence comprises PR-RT-RNAseH-INT, which js 10 kb long (Figure 5) (Sabot *et al.*, 2004; Wicker *et al.*, 2007). **3. *Athila* group.** They are similar to the gypsy group; however, their internal sequence is different from gypsy, and their LTR is longer, which is about 10-12 kb long

(Sabot *et al.*, 2004; Wicker *et al.*, 2007). **4. *Terminal-repeat retrotransposon in miniature (TRIMs)* group.** This group comprises short elements of 500 bp (Witte *et al.*, 2001). TRIMs possess a partial identity (100-250 bp long) with "LTRs" and are also called direct terminal repeats (TDRs) (Witte *et al.*, 2001). The large internal deletion generated a defective and non-autonomous element (Witte *et al.*, 2001). **5. *LArge Retrotransposons Derivatives (LARDs)* group.** They are the non-autonomous group for lacking *gag* and *pol* genes (Kalendar *et al.*, 2004). They contain a conserved sequence produced from the RNA secondary structure and possess a large LTR (about 4.5 kb) region (Kalendar *et al.*, 2004).

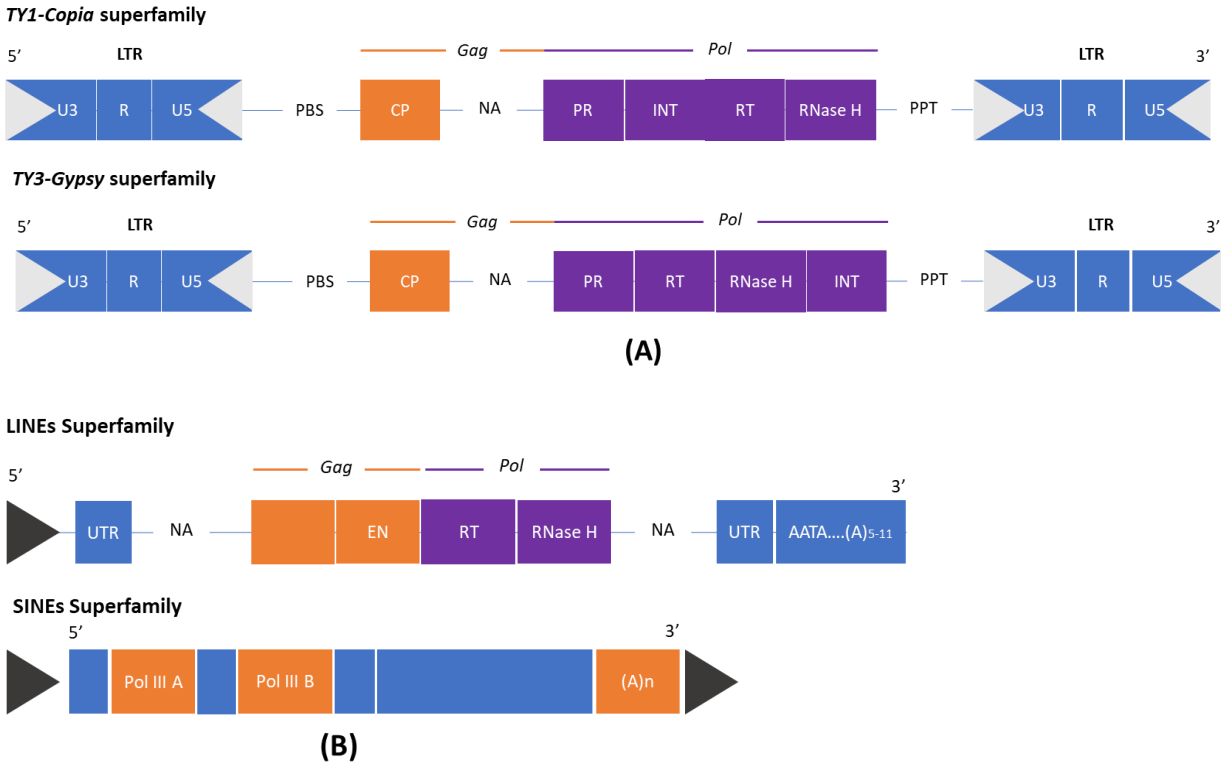

Figure 5. The representative structure of Class I Retrotransposon which consist of two subclasses, A. Subclass I and B. Subclass II. LTR: Long Terminal Repeat; U3: Unique 3RNA; R: Repeated RNA; U5: Unique 5 RNA; CP: Capside Protein; INT : Integrase; RT: Reverse Transcriptase; PBS: Primer Binding Site; PPT: PolyPurine Tract; NA: Nucleic Acid binding moiety; UTR: Untranslated Region; Pol III A and Pol III B: RNA Polymerase III promoter recognition site (Sabot et al., 2004).

Subclass II

Subclass II of the retrotransposons synthesize a newly reverse-transcribed DNA directly at the target site, known as retroposons (Sabot *et al.*, 2004). Unlike the first subclass, retroposons do not encode reverse transcriptase (Sabot *et al.*, 2004). They lack Terminal Inverted Repeats (LTRs) or Terminal Direct Repeats (TDRs), therefore considered as non-autonomous elements (Sabot *et al.*, 2004). Their structural region is different from retrotransposon; however, they have a protein region that shares a similar function as the first subclass (Sabot *et al.*, 2004).

Retroposons are divided into two groups, namely *Long interspersed Nuclear Element* (*LINEs*) and *Short Interspersed Nuclear Elements* (*SINEs*) (Table 2) (Sabot *et al.*, 2004).

LINEs elements contain two open reading frame one of which resembles Gag protein and another ORF resembles to Pol protein but lack protease and integrase enzymes (Dawson *et al.*, 1997; Volff *et al.*, 2001). The integration and reverse-transcription occur at the same time but at the target transposition region. *LINEs* are generally 1-8 kb long (Figure 5) (Jurka, 1998; Sabot *et al.*, 2004).

SINEs, as their name indicates, they are composed of a short sequence of about 100-300 bp long (Sabot *et al.*, 2004). These elements are lacking in the *transposase* gene (Sabot *et al.*, 2004). Although *SINEs* are non-autonomous elements, they are not mutated; instead, they are evolutionarily derived from RNA synthesized by pol II or pol III (Figure 5) (Kramerov & Vassetzky, 2005; Sabot *et al.*, 2004; Wicker *et al.*, 2007).

Table 2. Classification of Transposable Elements in Plants including class, subclass, group, species, and examples.

Class	Subclass	Group	Species	Examples
Class 1	LTR retrotransposon	*gypsy*	Rice	RIRE3, SZ, OSR
			Wheat	Fatima, Sabrina
			Barley	BAGY
			Maize	Reina, Grande
		copia	Rice	
			Wheat	Angela, Wis
			Barley	BARE-1
			Maize	
		athila	Wheat and barley	Wham, Sabrina
		LARDs	Wheat and barley	Sukkula
			Rice	Spip, Sqiq
		TRIM	Wheat and barley	Veju
	Non-LTR retrotransposon	*LINE*	Wheat and barley	Karine
		SINE	Wheat	Au
Class II	DNA transposon	*CACTA*	Wheat and barley	Casper, Jorge
		hAT	Maize	Ac/Ds, Mutator
		MuDR	Wheat and barley	Mutator
	MITEs	*Stowaway*	Wheat and barley	Hades, Fortuna
		MDM	Rice	MDM
	LITEs/FoldBack	NA	Wheat and barley	Apollon, Zeus
	Helitrons	NA	Maize	Helitron

Note 1. Refined from "Plant Transposable Elements, with emphasis on grass species" by (Sabot et al., 2004), Euphytica 139: 227–247

Transposable Elements (TEs) Class II

TEs Class II use DNA as an intermediate for transpositions via cut and paste mechanism or rolling circle mechanisms (Sabot *et al.*, 2004; Wicker *et al.*, 2007). The donor elements are replicated in the original sites and then transposed and integrated into the target location (Sabot *et al.*, 2004; Wicker *et al.*, 2007). As the first class of TEs, Class II elements also contain the autonomous and non-autonomous elements (Sabot *et al.*, 2004; Wicker *et al.*, 2007). The class II elements are divided into three groups, based on their transposition capability and length (Table 2) (Sabot *et al.*, 2004; Wicker *et al.*, 2007).

DNA-Transposon

The DNA transposons are divided into subclasses based on their different transposase coding sequences and the target site duplication (Capy, 1998). The transposase region structure is different, mainly because of the sequence homology and the presence or absence of DDE motif (two aspartates D followed by a glutamate E) (Capy, 1998). Based on their transposase coding sequence, DNA transposons could be classified into many superfamilies, including *CACTA*, hAT, and *MuDR* (Table 2) (Langdon *et al.*, 2003; Wicker *et al.*, 2007). During transposition, the transposase requires Terminal Inverted Repeat (TIR) sequence to initiate the excision before reintegrating into the target site (Kumar & Hirochika, 2001).

Miniature Inverted repeats Transposable Elements (MITES)

MITES have a small DNA sequence between 30 to 500 bp long flanked by TIRs at both ends (Sabot *et al.*, 2004). These elements are lacking in the coding sequence; therefore, they require a transposase donor for the transposition (Sabot *et al.*, 2004). It has been discovered that the source of transposase varies in the different species, for example, *PIF* transposase in Maize and *PONG* transposase in rice (Jiang *et al.*, 2003; Zhang *et al.*, 2004). Classification of MITEs is dependent on the source of the transposase enzyme (Lai *et al.*, 2005).

Helitrons: Rolling-circle transposons

Helitrons have a distinctive transposition process, which is occurred via a rolling-circle transposition mechanism. Helitrons use single-strand DNA cut. Unlike other DNA transposons, Helitrons do not generate target site duplication (TDSs) (Kapitonov & Jurka, 2001). The structure of Helitrons comprises of 5'-TC and CTRR-3' motif (where R is purine), a short hairpin of palindromic sequence (16-20 nucleotides) observed 11bp upstream from the 3' end (Sabot *et al.*, 2004; Wicker *et al.*, 2007). The internal sequence of Helitrons contains Y2-type tyrosine

recombinase with a helicase domain and replication initiator for transposition process (Sabot *et al.*, 2004; Wicker *et al.*, 2007).

2.7.1 Ac/Ds transposons

Ac and *Ds* are the first transposable elements found by McClintock when she studied the color variegation in maize kernels (McClintock, 1951). She discovered that in chromosome 9, a chromosome breaks frequently happen, which are associated with color variegation (McClintock, 1951). She subsequently found that a particular element plays a significant role in genome reorganization, named the *Dissociation* (*Ds*) element (McClintock, 1951). Later she found that the *Ds* element is dependent on another element, termed *Activator* (*Ac*), which harbors the transposase enzyme for the activation and transposition of *Ds* element to a different location (McClintock, 1951). The *Ds* insertion at the new genomic region could lead to a heritable genetic mutation (Turriziani-Colonna, 2014). Such mutations induce the mosaic color in corn seeds and leaves and have been observed with distinctive phenotypes in many crop species (Turriziani-Colonna, 2014).

Maize *Ac* element belongs to the *hAT* superfamily of the second group of DNA Transposable Elements (Sabot *et al.*, 2004). *Ac* element transposes by a cut and paste mechanism by recognizing 11 bp long unique terminal inverted repeats (Kunze & Weil, 2002). This process generates 8 bp target site duplication upon the insertion and integration of the *Ac* element into a new target region (Kunze & Weil, 2002). Maize *Ac* element comprises 4.6 kb DNA sequences encoding 3.5 kb open reading of the transposase (TPase) enzyme, 11-bp terminal inverted repeats (TIRs), and approximately 240 bp long essential subterminal sequences (Du *et al.*, 2011). *Ac* coding sequence has a transcription initiation site between 304 bp and 364 bp (Du *et al.*, 2011). Specific sequences (11 bp) have been observed at both 5' end (C/TAGGGATGAAA) and 3' end (TTTCATCCCTA) (Figure 6) (Du *et al.*, 2011; Kunze *et al.*, 1987; Kunze & Weil, 2002; Lazarow *et al.*, 2013).

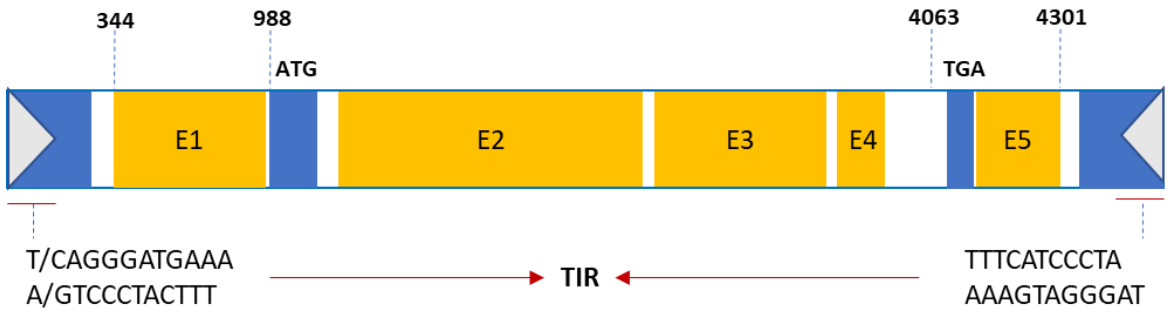

Figure 6. Structure of maize Activator (Ac) element. Transcription starts at 344 bp and ends at 4063 bp. The TPase start codon is at 988 bp. E1-E5: Exon 1-5 (Lazarow et al., 2013).

The Ds element lacks a transposase encoding region while retains TIRs and subterminal regions of 250-300 nucleotide, necessary for its transposition (Singh et al., 2006). The *Ds* element could move to other sites only in TPase enzyme presence (Du *et al.*, 2011). The *Ds* elements are wildly divergent and could be classified due to the frequency of causing the chromosome breakage (McClintock, 1947). One that causes the chromosome break at high frequency is *Ds1,* whereas other elements are less frequent, named *Ds2* (McClintock, 1947). *Ds1* element is ~400 bp long and shares 11 bp TIR and subterminal repeats with *Ac* element (McClintock, 1947). Whereas, *Ds2* element comprises a more extended sequence about ~ 1kb long and shares 200 bp with the *Ac* element at their end (McClintock, 1947). The deleted *Ac* sequence in the *Ds* element was replaced with an unrelated sequence (Merckelbach *et al.*, 1986; Peacock *et al.*, 1984). Subsequently. Other forms of *Ds* elements have been identified based on the sequence's variability (Figure 7) (Du *et al.*, 2011).

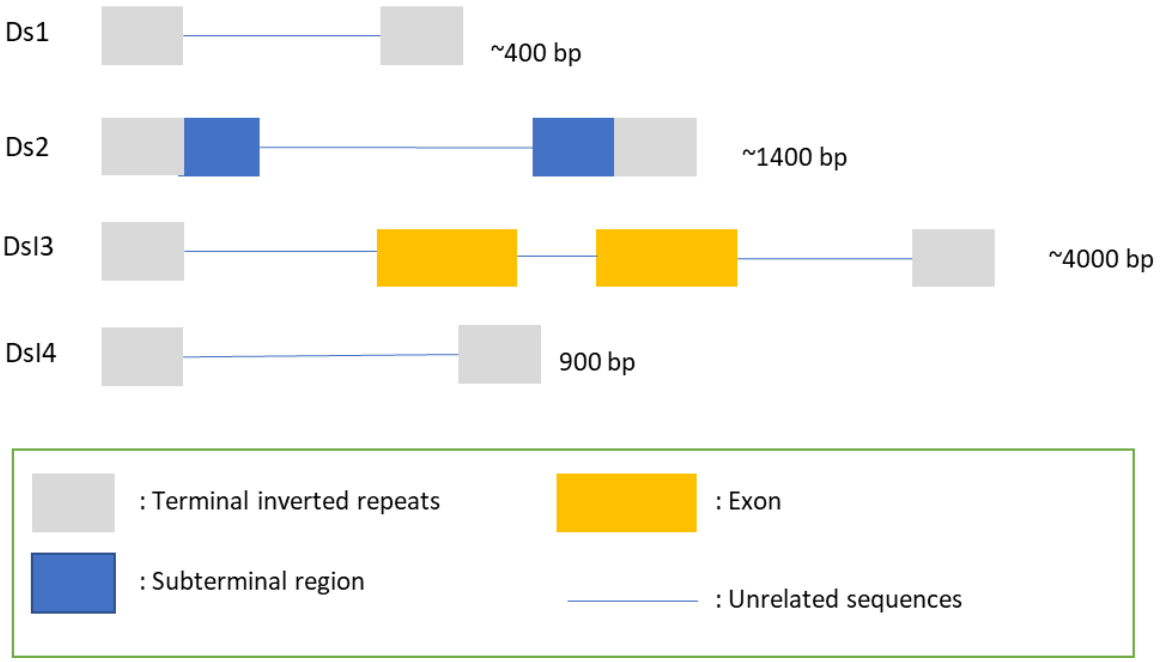

Figure 7. Structure of different types of Ds elements. Ds1 and DsI4 share with Ac element only inverted terminal region. Ds2 is about 1400 bp long, TIRs and subterminal region share similar sequences as of Ac element. DsI3 is the longest Ds element (4000 bp) and shares the same two exons and TIRs with Ac (Du et al., 2011).

2.7.2 Transposon as functional genomic tools

The *Ac*/*Ds* system offers valuable advantages over other techniques. The system is relatively fast and straightforward in comparison to, for instance, T-DNA insertion strategies, where a large number of transgenic plants are needed to identify genes (Wu *et al.*, 2019). Once the stable *Ac*/*Ds* plants are generated, new tagged lines could be generated by the conventional breeding approach (Wu *et al.*, 2019). It could reduce the tissue culture and tedious genetic transformation steps and make the functional genomics steps more efficient (Wu *et al.*, 2019). Furthermore, the preference of the *Ac*/*Ds* system to land into the linked genes makes this approach highly suitable to identify genes mapped near the transposon insertions (Koprek *et al.*, 2000; Osborne *et al.*, 1991).

Ac/*Ds* elements have been introduced into many crop species, including tomato (*Lycopersicon esculentum*) (Carter *et al.*, 2013; Yoder *et al.*, 1988), broccoli (*Brassica oleracea*)(Mckenzie & Dale, 2004), potato (*Solanum tuberosum*) (Knapp *et al.*, 1988; Lu *et al.*, 2014), carrot (*Daucus carota*), *Arabidopsis thaliana* (Van Sluys *et al.*, 1987), tobacco (Baker *et al.*, 1986), soybean (*Glycine max*)(Mathieu *et al.*, 2009), *Brachypodium distachyon (Wu et al., 2019),* wheat (Takumi, 1996), rice (*Oryza sativa*) (Guiderdoni & Gantet, 2012; Jiang *et al.*, 2003; Shimamoto *et al.*, 1993), maize (Li *et al.*, 2013) and barley (*Hordeum vulgare*) (Ayliffe *et al.*, 2007; Koprek *et al.*, 2000; Lazarow & Lütticke, 2009). Using this system, the function of few genes in barley

has been described, including the role of *WAK* genes in root elongation (Kaur *et al.*, 2013; Tripathi *et al.*, 2020), malting related genes (Singh & Singh, 2017; Singh *et al.*, 2012), and *miR172* induced spike architecture (Brown & Bregitzer, 2011; Tripathi *et al.*, 2018).

Ac/*Ds*-based activation tagging has also been introduced by including strong promoter or enhancer sequences, resulting in the overexpression of adjacent endogenous genes where Ac/Ds elements are landed (Ayliffe *et al.*, 2007). It has been introduced in some organisms, such as *Arabidopsis* (Wilson *et al.*, 1996) and barley (Ayliffe *et al.*, 2007). This approach can generate the mutant plant that mostly confers dominant genes and circumvents gene redundancy, especially in the crop species with large genomes (Ayliffe *et al.*, 2007; Wen *et al.*, 2004). This great benefit makes the activation tagging approach attractive and useful for functional genomics studies (Gu *et al.*, 2003; Wen *et al.*, 2004; Zhao *et al.*, 2001).

CONNECTING STATEMENT BETWEEN CHAPTER II & III

Molecular information about β-glucan biosynthesis is lacking due to the availability of limited genomic resources. Recently, *a Thaumatin Like Protein 8*, *TLP8*, has been identified in barley that interacts with β-glucan to regulate its content (Singh *et al.*, 2017). However, there is no report yet about the *TLP8* gene in oat. Therefore, in our project, we designed the experiment to retrieve and identify the oat *TLP8* gene and generate a TLP8 knock-down line to investigate β-glucan synthesis and regulation further. It will provide valuable information as the demand for oat production has been increasing these years due to its health-promoting properties, especially the fiber content.

We provided our experiment design in chapter III, including identifying the *TLP8* gene ortholog, designing an RNAi construct, particle gene gun bombardment, tissue culture system, and *TLP8* expression. Results and analysis are provided in the result and discussion part. Expression analysis conducted in T2 dry seeds of RNAi transgenic lines demonstrated that *TLP8* expression is significantly reduced compared to non-transgenic seeds, as expected. Chapter III is formatted as a publication manuscript. The co-authors are listed as follows: Annis Fatmawati, Wei-Yuan Chen, Rajvinder Kaur, and Jaswinder Singh. Each co-author's contribution is described in the Preface.

CHAPTER III : Functional Characterization of Genes Associated with β-glucan in Oat

3.1 Abstract

Oat is one of the major cereals that is grown worldwide for human food and animal feed. Its use in the human diet is becoming popular due to its ability to reduce serum cholesterol and glucose levels. It has been attributed to its high β-glucan content. β-glucan is a major non-starch polysaccharide, consisting of double β-1,3 and β-1,4 linkages. Information about β-glucan synthesis in oat is lacking due to the availability of limited genomic resources. Recently, *TLP8* has been identified in barley that interacts with β-glucan to regulate its content in grain. Higher transcript abundance of *TLP8* in barley grains reflect lower amounts of β-glucan, and vice-versa. We hypothesize that the downregulation of *TLP8* could increase β-glucan content in oat. The *TLP8* ortholog in oat was retrieved, and an RNAi construct was created. The genetic transformation was then conducted via the bombardment gun method. Transformants were generated and selected using a *Bialaphos Resistance* (Bar*)* marker gene, yielding a significant putative calli frequency (49.8%), with transformation efficiency up to 7.4% in T0 stages. Histochemical assays confirmed the expression of *the Bar* gene. Calli transformed with the *Bar* gene were resistant to Bialaphos 3mg/L, and transgenic plants were resistant to herbicide glufosinate LIBERTY (0.2%). Two transgenic events were advanced to T1 generation. Expression analysis conducted in T2 seeds demonstrated that *AsTLP8* expression is significantly reduced in transgenic seeds than non-transgenic seeds. Interestingly, transgenic seeds were germinated faster than the non-transgenic ones, which requires further investigation. In conclusion, transgenic lines for *AsTLP8* could lead to a better understanding of β-glucan synthesis and regulation in oat.

3.2 Introduction

β-glucan is a component found characteristically in the Poaceae grains family, which is not present in dicotyledons and most other monocotyledons cell walls (Burton *et al.*, 2006). Besides, β-glucan varied depending on the species; it might consist of zero to more than 45% (Burton & Fincher, 2014). Notably, some cereals, such as barley and oat, contain a large amount of β-glucan, making them substantial dietary crops (Mantovani *et al.*, 2008). β-glucan is highly beneficial due to its health-promoting activities to prevent cardiovascular disease, high serum cholesterol, obesity, and non-insulin-dependent diabetes (Anttila *et al.*, 2004). Besides, β-glucan has an antitumor, antimicrobial, anti-fungal, antimutagenic, and

immunostimulant activity (Mantovani *et al.*, 2008). β-glucans are comprised of an unbranched and unsubstituted chain of glucosyl residues through the 1.3 and 1.4 linkages (Harris & Fincher, 2009). The β-glucans complex predominantly constitutes two or three 1.4 linkages separated by 1.3 linkages, namely cellotriosyl and cellotetraosyl units (Harris & Fincher, 2009). Even though the structure, component, and biological properties of β-glucan are well characterized, the information of enzymes and genes regulating β-glucan biosynthesis and regulation are poorly identified.

Cellulose Synthase (*CESA*), *Glucan Synthase-Like* (*GSL*), and *Cellulose Synthase-Like* (*CSL*) gene families have been recognized to be involved in the most β linked-polysaccharide synthesis (Doblin *et al.*, 2009; Kaur *et al.*, 2017; Kaur *et al.*, 2016). The *CESA* families constitute several individual genes that independently encode a catalytic subunit of cellulose or callose (β-1,3-glucan) synthesis (Delmer, 1999; Farrokhi *et al.*, 2006). The *GSL* gene is also required for callose synthase activity (Brownfield *et al.*, 2007; Li *et al.*, 2003). Whereas the *CSL* genes are widely known to encode the enzymes for the background of non-cellulosic wall polysaccharides (Farrokhi *et al.*, 2006). However, these genes contribute only a part of total β-glucan content, as reported elsewhere (Newell *et al.*, 2012). Besides, given a low level of β-glucan in *Arabidopsis thaliana CSL* overexpressed transformants (Burton *et al.*, 2006; Doblin *et al.*, 2009), it is concluded that other coding genes, enzymes, or cofactors probably involved in β-glucan synthesis and regulation (Burton *et al.*, 2006).

Recently, the barley *Thaumatin-Like Protein 8* (*TLP8*) gene has been reported to associate malting quality in barley (Singh *et al.*, 2017). TLP8 is highly expressed in malting varieties that correlates with a lower amount of β-glucan (Singh *et al.*, 2017). In contrast, a lower expression of *TLP8* was seen in high β-glucan feeding varieties (Singh *et al.*, 2017). *TLP8* harbored a carbohydrate-binding motif (CQTGDCGG), which binds to insoluble β-glucan in the redox-regulated interaction (Singh *et al.*, 2017). This bond probably assists the β-glucan degradation from the cell wall, helpful during the malting process (Singh et al., 2017).

Nevertheless, reports yet to emerge about the function of *TLP8* in oat β-glucan synthesis. In this experiment, we downregulated the *TLP8* gene using the RNA interference approach in *Avena sativa* var. park. RNA interference was chosen due to its success story to downregulate genes in many organisms such as barley (Gubler *et al.*, 2008; Zalewski *et al.*, 2010), wheat (Travella *et al.*, 2006; Yan *et al.*, 2004), rice (Ashikari *et al.*, 2005), and Arabidopsis (Kerschen *et al.*, 2004). Our experiment aimed to generate the knocked-down *TLP8* transformants for

their subsequent use in the analysis and correlation of *TLP8* with β-glucan biosynthesis and regulation.

3.3 Materials and Method

3.3.1 In-silico Identification of Oat *TLP8*

Barley *TLP8* (*HvTL8*) was used as a query sequence to carry out nucleotide blast (BLASTn) to identify the candidate *TLP8* gene in oat. The genes were retrieved from an in-progress exome capturing effort (Langdon and Tinker, personal communication) of diploid oat with A genome, which was also confirmed by comparing its sequence with the published *A. atlantica* genome (Maughan Peter *et al.*, 2019). Primer was then designed to validate the candidate genes.

3.3.2 Generation of RNAi Construct

The pMCG161 binary vector (Figure 8) (http://www.chromdb.org/mcg161.html) was chosen to construct the hpRNAi cassette. The cassette is composed of the *Bar* gene under the regulatory control of ubiquitin promoter and nos terminator. The cassette for cloning the RNAi construct consisted of two restriction sites separated with a rice waxy intron driven by the cauliflower mosaic virus (CaMV) 35S promoter and maize Adh1 intron.

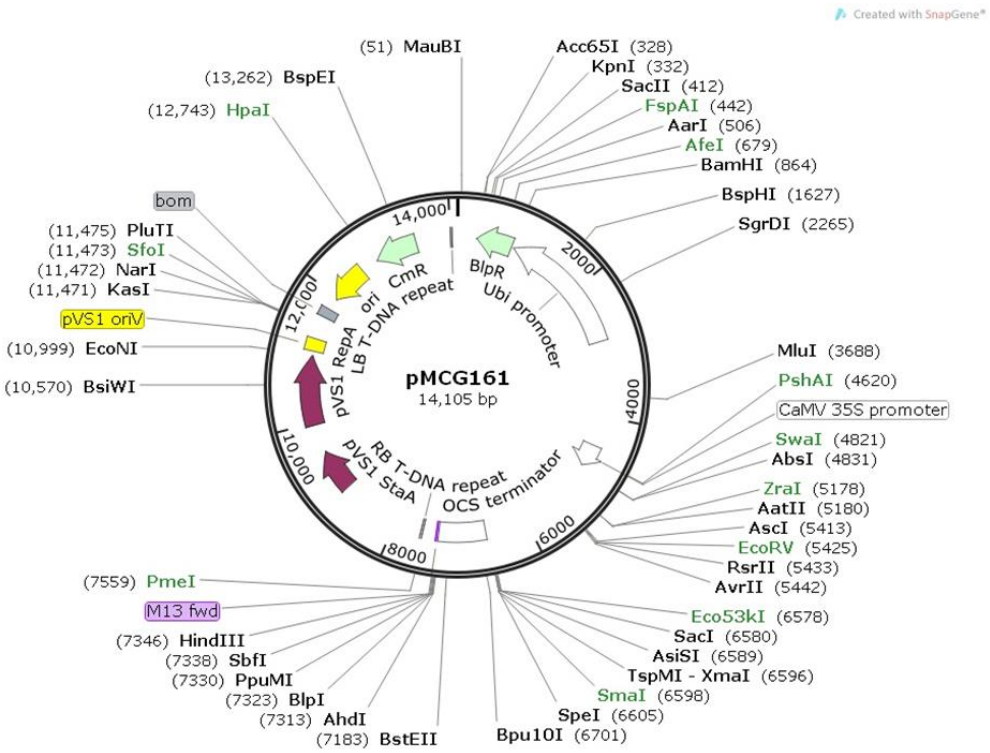

Figure 8. The schematic figure of pMCG161 binary vector

The RNAi cassette contained sense and antisense of 304 bp *AsTLP8* (Figure 9). Firstly, the *AsTLP8* fragment was cloned into pGEMT and amplified with AsTLP8rsF and AsTLP8rsR

primers (Table 6) containing the restriction sites for cloning: *SacI* and *SpeI*, and *RsrII* and *AvrII*. The fragments were then cut and cloned in the sense and antisense orientation into the pMCG161 vector. It was then transformed to the DH5α strain of *Escherichia coli* and selected on LB medium containing 35 mg/L chloramphenicol. The colony survived was then confirmed by the restriction analysis and sent for sequencing.

Figure 9. The schematic representation of pMCG161-AsTLP8 cassette. The expression of the RNAi cassette was under CaMV 35S promoter and terminated at Ocs terminator. The selection marker chosen was the Bar gene under UBI promoter and Nos terminator.

3.3.3 Transformation to *E. Coli*

The plasmid was transformed to DH5α strain of *E. Coli* for its replication following Chan *et al.* (2013). The DHSα was taken out from -86^{0}C and thawed on ice for approximately 20-30 minutes. The ligation reaction as much as 1 -5 µl was then mixed into 20 – 50 µl of competent cells in a tube. Afterward, the mixture was gently flicked a few times and incubated on ice for 20 – 30 minutes. Heat shock was then applied by putting the mixture in a water bath at precisely 42^{0}C for 30 – 60 seconds. Then it was put back into ice for 2 minutes. The LB medium as much as 950 µl (without antibiotics) was added and grown in a 37^{0}C shaking incubator for 45 minutes. It was then plated onto the LB agar plate containing the antibiotic, followed by incubation at 37^{0}C overnight. The day after, the positive colony was picked and sub-cultured in liquid LB medium.

3.3.4 Plasmid Isolation

Following (Ehrt & Schnappinger, 2003), 200 µl of solution I containing 50 mM glucose, 10 mM EDTA, and 25 mM Tris pH 8.0 was added to a plasmid pellet and incubated for 10 minutes on ice. Solution II constitutes 10 N NaOH and 10% SDS was then added as 300 µl and incubated for 5 minutes. It was followed by adding solution III (potassium acetate and glacial acetic acid) and incubating for 5 minutes. The centrifugation was then performed at 13000 rpm for 10 minutes at room temperature. As much as 700 µl of supernatant was moved to a 1.5 ml tube and added 4 µl of RNAse followed by incubation in 1 hour. Chloroform was added to the solution as 700 µl and centrifuged at the highest speed for 2 minutes. Afterward, isopropanol was added and centrifuged at the highest speed for 10 minutes. The pellet was washed by applying 70% ethanol. The solution was then centrifuged for 5 minutes and poured off. The final step was adding the molecular water as 30 µl.

3.3.5 Plant Materials and In Vitro Culture

Mature seeds of hexaploid (*Avena sativa*) var. Park were surface sterilized in 20% bleach for 20 minutes, followed by 3-4 rinses in sterile distilled water. The tissue culture system was carried out according to (Cardinal *et al.*, 2016).The sterilized seeds were subjected to a regeneration medium, using MS-basal medium (Murashige & Skoog, 1962), supplemented with sucrose 30 mg/L, thiamine HCL 1 mg/L, pyridoxine HCL 0.5 mg/L, nicotine acid 0.5 mg/L, CuSO4 0.16 mg/L, Indoleacetic acid (IAA) 1 mg/L, 6-benzyl amino purine (BAP) 1 mg/L hormone, and solidified Gelzan 2.8 g/L (Table 3). The medium was set to pH 5.8 and autoclaved at 121^0C for 20 minutes. The plates were incubated for 3-4 days in 16 hours light and 8 hours dark system. Germinated shoot and root were manually removed. The embryogenic part was then placed in a callus induction medium (DBC3), a second-step of calli induction medium, consisted of MS basal medium, maltose 30 g/L, casein 1g/L, myo-inositol 0.25 g/L, proline 0.69 g/L, thiamine HCL 1 mg/L, $CuSO_4$ 5 µM, and the combination of 2.4-dichlorophenoxyacetic acid (2.4-D) 1 mg/L and BAP 0.5 mg/L (Table 3). The samples were then incubated for two weeks to generate green calli. To maintain it, calli were sub-cultured every two weeks in DB3 induction medium.

Table 3. Composition of the different medium using for tissue culture and transformation

Component	Regeneration Medium	Callus induction medium (DBC3)	Osmotic medium	Rooting medium
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	-	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
Nicotine acid	0.5 mg/l	-	-	0.5 mg/l
CuSO4	0.16 mg/l	5 µM	5 µM	0.16 mg/l
2,4-D	-	1 mg/l	1 mg/l	-
BAP	1 mg/l	0.5 mg/l	0.5 mg/l	-
IAA	1mg/l	-	-	1 mg/l
IBA	-	-	-	1 mg/l
NAA	-	-	-	1 mg/l
Gelzan	2.8 g/l	2.8 g/l	2.8 g/l	2.8 g/l
pH	5.8	5.8	5.8	5.8
Mannitol	-	-	36.43 g/l	-
Sorbitol	-	-	40.038 g/l	-

3.3.6 Particle Gene Gun Bombardment

The Bio-Rad Biolistics PDS-1000/He was used for the bombardment of AsTLP8.RNAi constructs into green calli of *Avena sativa*. Calli were firstly subjected to an osmotic medium for 4 hours before the bombardment and 24 hours after that. It aimed to increase the DNA uptake, thus increases the transformation efficiency (Cardinal et al., 2016). The osmotic medium is a callus induction medium (DBC3) without maltose and a 0.2 mannitol and a 0.2 sorbitol (Table 3). The calli bombarded were placed in a 16h light and 8h dark system at 25^0C (Able et al., 2001). For the transformation, the methodology developed by Cardinal *et al.* (2016) and Kikkert *et al.* (2005) was followed. 12 µg/L DNA of AsTLP8.RNAi plasmid was used for six plates. The DNA-coated gold microcarriers were prepared before the shooting. Firstly, 36 µl of gold suspension was taken into a new tube and followed by centrifugation for 30 seconds at high speed. The supernatant was then removed, and 200-300 µl of water was added slowly, followed by centrifugation for 3 minutes at 2000 rpm. The plasmid DNA was then added into the tube and gently tapped. Sterile water was added to complete 250 µl with the DNA. The other solution, 250 µl $CaCl_2$ and 50 µl (0.1M) spermidine, were added. The solution was mixed by tapping, followed by vortex, which then kept on ice for 30 minutes with mixing every 10 minutes. It was then centrifuged for 5 minutes at high speed. The supernatant was removed. It was then washed by 200-300 µl ethanol absolute followed by centrifugation for 1-2 minutes with high speed. After removing supernatant, the 36-60 µl of ethanol absolute was added. Six µl of precipitation solution was dispensed into the microcarrier discs and let to dry for 5-10 minutes. The bombardment was performed using an 1100 psi rupture disk (Bio-Rad). The vacuum was switched to 25-28 mm Hg, and target calli were bombarded.

3.3.7 Selection of Putative Transgenic Calli

The calli were incubated overnight at 16 hours light and 8 hours dark system in the osmotic medium after bombardment. The calli were then sub-cultured on to the selection medium, a DBC3 medium with 3 mg/L of Bialaphos (IGNITE, Bayer Crop, Canada). Bombarded tissue was incubated for two weeks in the first selection medium plate. The survived green calli were sub-cultured into the second and followed by the third selection plate. While the dead calli were discarded.

3.3.8 Regenerating Plants

Following the selection for 2-3 rounds, calli were sub-cultured to regeneration medium as mentioned above (Table 3). When the shoot was formed about 2-3 cm, it was sub-cultured to

a rooting medium consisting of a basal MS medium, sucrose 30 g/L, thiamine HCL 1 mg/L, pyridoxine HCL 0.5 mg/L, nicotine acid 0.5 mg/L, CuSO4 0.16 mg/L, IAA 1 mg/L, NAA 1 mg/L, and NBA 1 mg/L (Table 3). The plants with germinated root and shoot were then moved to the soil.

3.3.9 Basta Leaf Painting

The basta painting was performed by applying commercial herbicide glufosinate-ammonium liberty (0.2%) (IGNITE, Bayer Crop, Canada) in young leaf plants. The examination of leaf performance was conducted one week after the assay. Plants with green and healthy leaves were considered as putative transgenic plants conferring the *Bar* gene.

3.3.10 Genomic DNA Extraction

Before performing the molecular characterization, the DNA of putative transgenic plants was extracted using the urea extraction method (Chen & Dellaporta, 1994). The young leaf samples were collected and quickly transferred to liquid nitrogen for immediate extraction. Samples can also be stored at -80^{o}C for further use. The leaf was ground using Tissue Lyser 2 Qiagen Apparatus to gain a fine powder. After that, 0.5 ml of urea buffer containing 5M NaCl, 2M Tris-HCL pH 8.0, 0.5 EDTA pH 8.0, and sarkosyl (N-laurylsarcosine) was added and mixed well. 0.5 ml phenol: chloroform (1:1) were added and mixed on a tabletop shaker for 1 hour. After the incubation, samples were centrifuged for 6 minutes. The supernatant was moved into a new tube and added with 1/10 vol 4.4M NH_4Ac and an equal volume of isopropanol. The tube was then inverted several times to precipitate the genomic DNA. The samples were centrifugated for two minutes, followed by a washing step with ethanol 70% and 90%. Pellet was then resuspended in 50 µl TE.

3.3.11 Molecular characterization

The different sets of primers were used to detect RNAi insertion in the putative transgenic plants, comprised of a pair of ZmAdh1.F1 and OsWaxy-A.R1, OsWaxy-A.F1 and OCS_Ter.R1, AF-7 and AF-9, and AF-2 and AF-3 (Table 6). The PCR reaction was comprised of 10 µl GoTaq Green Master Mix (Promega Corporation, Canada), 7 µl of molecular water, 1 µl of DNA sample, and each 1 µl for forward and reverse primer. The PCR condition was 94^{0}C in 30 seconds for denaturation, 30 seconds for annealing in a particular temperature based on the primer pair, and 72^{0}C for elongation in 1 minute. An agarose gel 1 % in TBE buffer 1% was used to perform gel electrophoresis.

3.3.12 RNA isolation and cDNA synthesis

The mature oat seeds were used in RNA extraction. The seeds were submerged in liquid nitrogen immediately to prevent RNA degradation. The samples were ground in a mortar to yield a fine powder by applying liquid nitrogen in it. The total RNA was extracted with the Total RNA Purification Kit (SIGMA) following the manufactures' recommendation. The RNA concentration was measured with an ND-1000 spectrophotometer. To perform cDNA synthesis, 1µg RNA treated using DNAse was reversed transcribed with iScript cDNA synthesis kit (Bio-Rad). Six µl of RNA was used as a template. To make a 20 µl reaction, 10 µl master mix, 3 µl oligo dT primer, and 1 µl enzyme were mixed. The amplification condition performed was 42^0 C for 5 minutes, 55^0C for 15 minutes, and 95^0C for 5 minutes. The cDNA was stored at -80^{o}C for the next PCR or directly used for PCR experiment.

3.3.13 RT-PCR analysis of *TLP8*

To check the endogenous *TLP8* gene expression, RT-PCR was performed. The RT-PCR was conducted using GoTaq® G2 Green Master Mix. A primer pair of AsTLP8rsF and AsTLP8rsR were used for the amplification (Table 6). The PCR was done at 95°C for 2 minutes, followed by 30 cycles at 95°C for 30 seconds, 63°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes. *β-ACTIN* was used as an expression control (Kaur *et al.*, 2013). For *β-ACTIN* amplification, AsActinF and AsActinR primers were used (Table 6).

3.3.14 qRT-PCR analysis of TLP8

qRT-PCR was performed as described by Singh *et al.* (2017). It was conducted using two biological and three technical replications for each sample. Relative expression was conducted with *TATA-Binding Protein2* (*TBP2*) as a reference gene (Ruduś & Kępczyński, 2018) and Brilliant III SYBR Green QPCR master mix (Agilent Technologies). Amplification was conducted in a 20-µL reaction mixture containing 160 nmol for each primer, 1× Brilliant III SYBR Green QPCR master mix, 15 µM ROX reference dye, and 0.3 µL of cDNA template. The amplification conditions were 95 °C for 10 min (hot start), followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The primer used for qRT-PCR were AfTBP2F and AfTBP2R for reference gene amplification and qASTLP8F and qAsTLP8R for target gene amplification (Table 6).

3.3.15 Statistical Data Analysis

Independent Student's *t*-test was performed to detect statistical differences of *AsTLP8* expression between transgenic seeds and non-transgenic seeds during a different time of imbibition. P-value of <0.05 was taken as the level for significance.

3.3.16 Examination of Seeds Under Selected Regeneration Medium

Seeds were subjected to the regeneration medium containing Bialaphos 3 mg/L to examine and confirm the *Bar* gene expression in the RNAi fragment insertion. If the gene is expressed, seeds could survive under the selection; in reverse, seeds would not be germinated. The examination was conducted before seeds imbibition and at 72 hours after imbibition.

3.3.17 Comparison of Germination Stages Between Transgenic and Non-transgenic Plants

The phenotypic differences were observed between transgenic plants and non-transgenic plants at the germination stages. After 96 h of imbibition, both plants were examined. The different traits, such as the development of shoot, development of root, and length of both organs were scrutinized and compared.

3.4 Results

3.4.1 The Ortholog *TLP8* Gene Retrieval

It was discovered that there were two scaffolds from diploid oat annotated with exons that match barley *TLP8* (*HvTLP8*). One annotation was found on scaf0283715, which came from a transcript in a salt-stressed library. It showed 86% (527/612bp) of sequence identities with 1% sequence gaps (Figure 10). Another annotation was on scaf0275258 retrieved in 3 exons in which the third exon matched with HvTLP8. The sequence shared 87% (396/457bp) identities, with 1% sequence gaps (Figure 11). The sequences are displayed below.

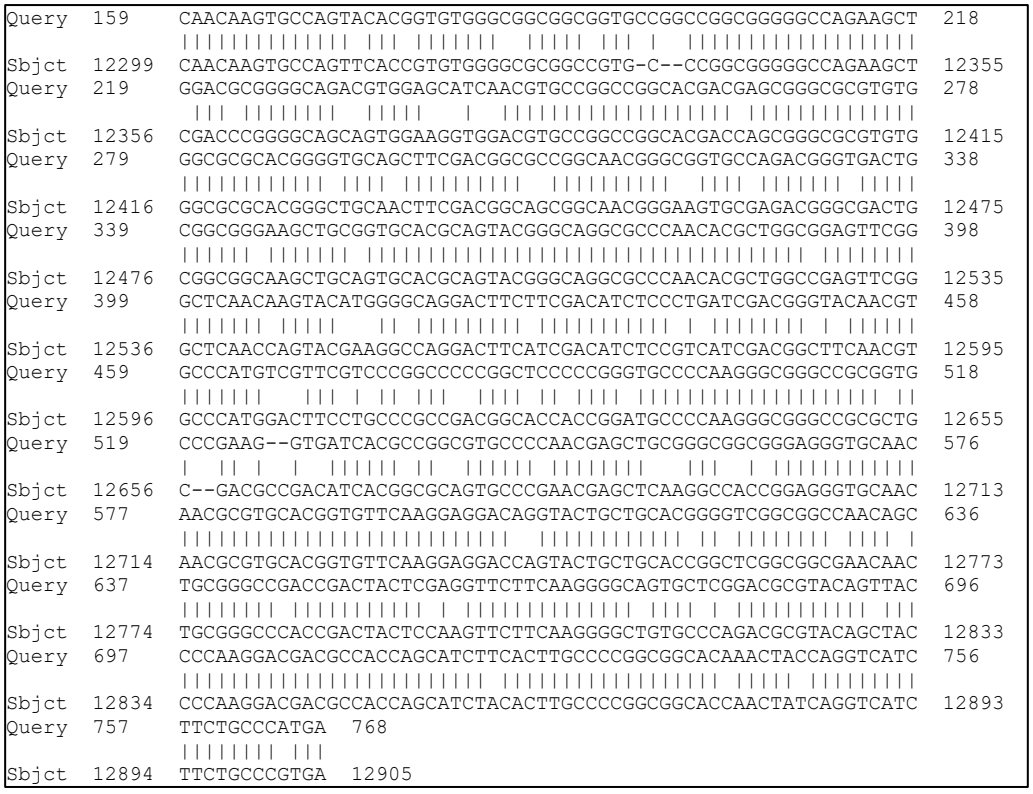

Figure 10. The sequence alignment of HvTLP8 as query and oat TLP8 candidate gene as a subject on scaf0283715.

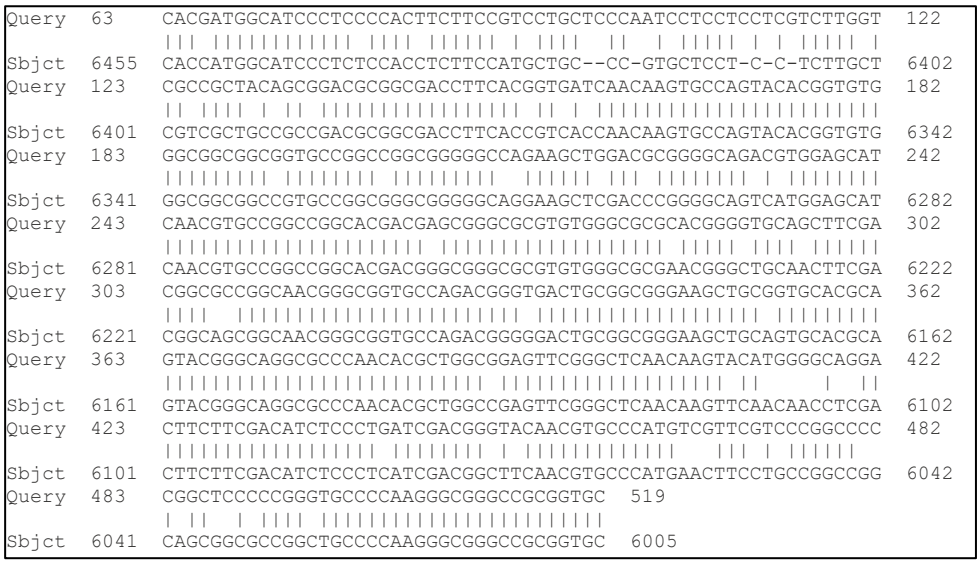

Figure 11. The sequence alignment of HvTLP8 as query and oat TLP8 candidate gene as a subject on scaf0275258.

3.4.2 The AsTLP8.pMCG161 binary vector construction

The *AsTLP8* cDNA was amplified using a pair of primers with restriction sites at the 5' flanked forward and 3' reverse primer. AsTLP8_rsF forward primer consists of RsrII and SpeI restriction sites; whereas, AsTLP8_rsR primer has AvrII and SacI restriction sites. The fragment was then extracted and ligated into the pGEMT cloning vector before introducing it into the expression vector pMCG161. It aimed to produce a large number of the vector by reproducing

and propagating it in bacteria *Escherichia coli*. The ligation was confirmed using the AvaII restriction enzyme. The colonies lane 1,2,3,4 were positive. The band's different pattern seen in colonies 1,3 and colonies 3,4 was due to different insertion orientations (Figure 12).

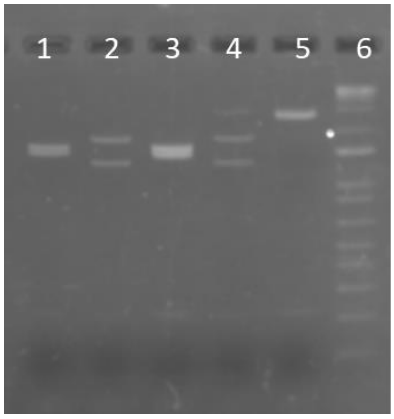

Figure 12. The confirmation of AsTLP8 ligation into pGEMT cloning vector. Lane 1,2,3,4 are the colonies with the inserted DNA, lane 5 is control (pGEMT), and lane 6 is a ladder.

Afterward, the pGEMT+DNA was digested using SacI and SpeI restriction enzyme to produce the first DNA insertion. The digestions were checked on gel electrophoresis, and an expected 289 bp fragment was created (Figure 13). It was then inserted into the pMCG161 vector and confirmed with PCR using primer OsWaxy-A.F1 and OCS_Ter.R1. The pMCG161 vector with DNA insertion amplifies the 775 bp fragment. While pMCG161 without the insertion amplifies 494 bp fragment (Figure 13). This information indicated the success of the first fragment insertion.

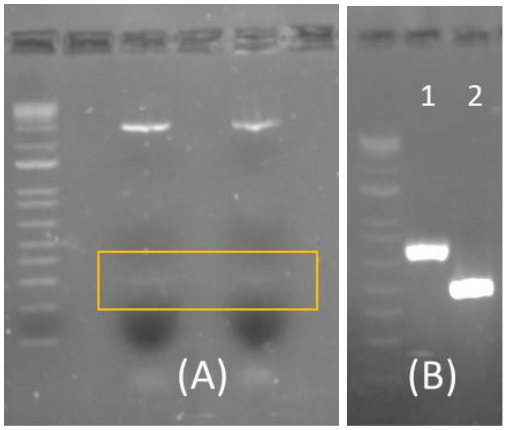

Figure 13. Confirmation of the first DNA insertion. A. The digestion of pGEMT+DNA using SacI and SpeI. B. PCR confirmation of the first insertion; 1. pMCG161+DNA and 2. Control (pMCG161).

For the second insertion, the pGEMT+DNA was digested with RsrII and AvrII. The restriction was confirmed by the band on 300 bp size, as shown in Figure 14A. The fragment was then introduced into pMCG161, and final confirmation was made using BamHI and PstI digestion.

Lane 3 and 4 were the colonies with the double insertion of *AsTLP8* (Figure 14 B). It showed that a final construct was digested at 7050 bp, 4734 bp, 1400 bp, 884 bp, and 581 bp.

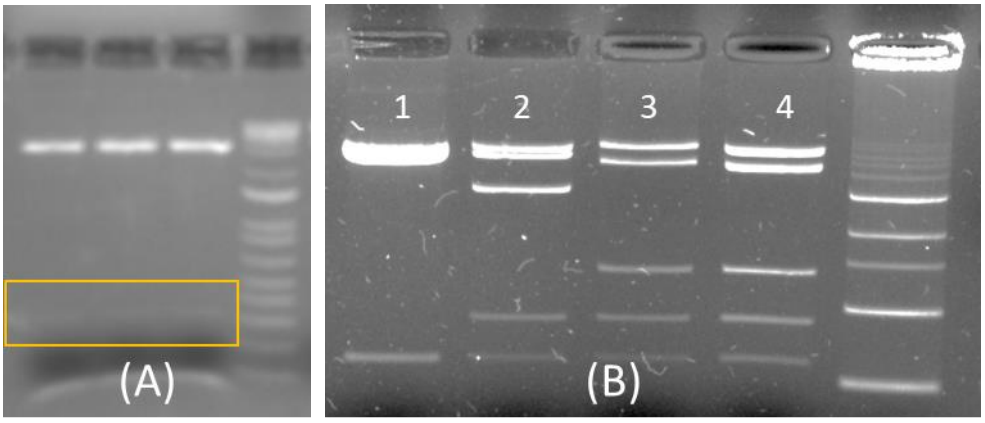

Figure 14. Final vector construction A. The digestion of pGEMT+DNA using RsrII and AvrII. B. Confirmation of final RNAi construct; 1. pMCG161, 2. Inter.pMCG161, 3 &4. AsTLP8.pMCG161.

3.4.3 Sequencing of *AsTLP8*.pMCG161 construct

For the final confirmation, the vector was sequenced at the Genome Quebec Sequencing facility. The sense RNAi sequence was determined with primer Os-waxy-AR.1 and confirmed that the RNAi sense transgene sequence was perfectly matched with the database (Figure 15). Likewise, the antisense transgene sequence was determined with the OsWaxy-A.F1 primer and showed a perfect alignment with the database (Figure 16). It explained that the AsTLP8.pMCG161 construct was successfully generated.

```
DATABASE        GGCGCGCCAGAGATATCAGACGGACCGACTAGTCCGGCGGGGGCCAGAAGCTCGACCCGG  60
CONSTRUCT       GGCGCGCCAGAGATATCAGACGGACCGACTAGTCCGGCGGGGGCCAGAAGCTCGACCCGG  60
                ************************************************************

DATABASE        GGCAGCAGTGGAAGGTCGAGGTGCCGGCCGGCACGACCAGCGGGCGCGTGTGGGCGCGCA  120
CONSTRUCT       GGCAGCAGTGGAAGGTCGAGGTGCCGGCCGGCACGACCAGCGGGCGCGTGTGGGCGCGCA  120
                ************************************************************

DATABASE        CGGGCTGCAACTTCGACGGCAGCGGCAACGGGAAGTGCGAGACGGGCGACTGCGGCGGCA  180
CONSTRUCT       CGGGCTGCAACTTCGACGGCAGCGGCAACGGGAAGTGCGAGACGGGCGACTGCGGCGGCA  180
                ************************************************************

DATABASE        AGCTGCAGTGCACGCAGTACGGGCAGGCGCCCAACACGCTGGCCGAGTTCGGGCTCAACC  240
CONSTRUCT       AGCTGCAGTGCACGCAGTACGGGCAGGCGCCCAACACGCTGGCCGAGTTCGGGCTCAACC  240
                ************************************************************

DATABASE        AGTACGAAGGCCAGGACTTCATCGACATCTCCGTCATCGACGGCTTCAACGTGCCCATGG  300
CONSTRUCT       AGTACGAAGGCCAGGACTTCATCGACATCTCCGTCATCGACGGCTTCAACGTGCCCATGG  300
                ************************************************************

DATABASE        ACTTCCTGCCCGGAGCTCCCTAGG    324
CONSTRUCT       ACTTCCTGCCCGGAGCTCCCTAGG    324
                ************************
```

Figure 15. A sequence alignment of construct AsTLP8.pMCG161 with the database in sense orientation, determined with Os-waxy-AR.1. The database is a retrieved TLP8 sequence, and the construct is an extracted AsTLP8.pMCG161.

```
DATABASE        GAGCTCCGGGCAGGAAGTCCATGGGCACGTTGAAGCCGTCGATGACGGAGATGTCGATGA  60
CONSTRUCT       GAGCTCCGGGCAGGAAGTCCATGGGCACGTTGAAGCCGTCGATGACGGAGATGTCGATGA  60
                ************************************************************

DATABASE        AGTCCTGGCCTTCGTACTGGTTGAGCCCGAACTCGGCCAGCGTGTTGGGCGCCTGCCCGT  120
CONSTRUCT       AGTCCTGGCCTTCGTACTGGTTGAGCCCGAACTCGGCCAGCGTGTTGGGCGCCTGCCCGT  120
                ************************************************************

DATABASE        ACTGCGTGCACTGCAGCTTGCCGCCGCAGTCGCCCGTCTGGCACTGCCCGTTGCCGCTGC  180
CONSTRUCT       ACTGCGTGCACTGCAGCTTGCCGCCGCAGTCGCCCGTCTGGCACTGCCCGTTGCCGCTGC  180
                ************************************************************

DATABASE        CGTCGAAGTTGCAGCCCGTGCGCGCCCACACGCGCCCGCTAGTCGTGCCGGCCGGCACGT  240
CONSTRUCT       CGTCGAAGTTGCAGCCCGTGCGCGCCCACACGCGCCCGCTAGTCGTGCCGGCCGGCACGT  240
                ************************************************************

DATABASE        CGATCTGCCACTGCTGCCCCGGGTCGAGCTTCTGGCCCCCGCCGGACTAGT  291
CONSTRUCT       CGATCTGCCACTGCTGCCCCGGGTCGAGCTTCTGGCCCCCGCCGGACTAGT  291
                ***************************************************
```

Figure 16. A sequence alignment of construct AsTLP8.pMCG161 with the database in antisense orientation, determined with Os-waxy-AF.1. The database is a retrieved TLP8 sequence, and the construct is an extracted AsTLP8.pMCG161.

3.4.4 Selection of Putative Transgenic Calli

After the bombardment of *AsTLP8*.pMCG161 vector into oat calli, putative transgenic calli were selected under the exposure of Bialaphos herbicide 3mg/L. As shown in Figure 17 A, most of the bombarded calli were survived after selection compared to non-transformed calli, which ultimately died. Out of 408 initial calli bombarded, 203 were survived, demonstrating 49.8% putative transgenic calli frequency (Table 4). It demonstrates that most of the calli harbored the *Bar* gene, which is resistant to Bialaphos herbicide. Similar phenomena were observed in other crops, such as maize, barley, wheat, and rice (Bhati *et al.*, 2016; McGinnis *et al.*, 2005; Nakamura *et al.*, 2010; Tingay *et al.*, 1997).

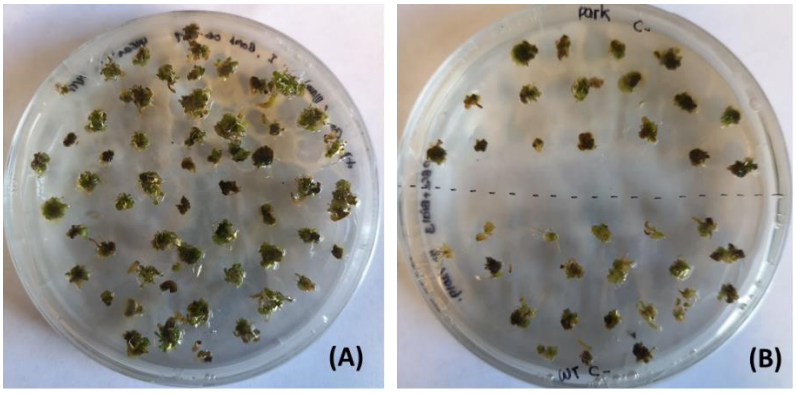

Figure 17. Calli were performed under Bialaphos herbicide 3mg/L. A. Putative transgenic Avena sativa calli, and B.Non-bombarded calli.

3.4.5 Basta Leaf Painting

The basta painting was performed in T0 and T1 plants by applying commercial herbicide glufosinate liberty (0.2%) in young leaf plants. Putative transgenic plants exhibited substantially less herbicide symptoms (mild necrosis) than control plants that showed necrosis in all areas applied with the herbicide, as shown in Figure 18. It represented that the putative transgenic plants harboring the *Bar* gene were also resistant to the application of glufosinate herbicide.

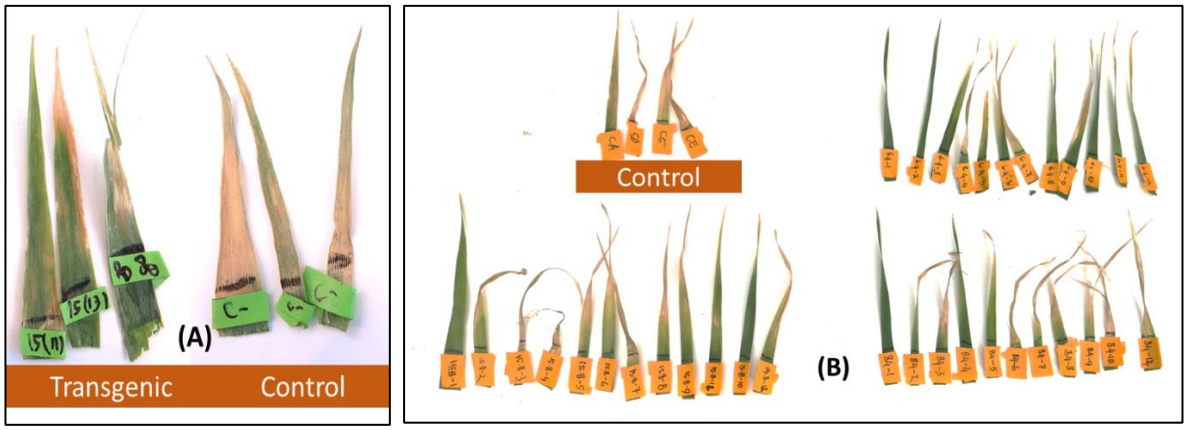

Figure 18. Basta leaf painting was performed in putative transgenic plants and non-transgenic plants in T0 (A) and T1 (B) under the application of 0.2% herbicide glufosinate liberty.

3.4.6 Molecular Characterization in T0 Plants

After plants were regenerated, molecular characterization was performed to identify the insertion of genes into the genome of *Avena sativa*. The PCRs were conducted to check the insertion of RNAi transgene in the sense orientation (primer AF2 and AF3) and antisense orientation (primer OsWaxy-A.F1 and OCS_Ter.R1) (Table 6). Out of 203 plants regenerated, 28 plants amplified at 532bp for the sense transgene insertion, and 18 of them were amplified at 775bp for the antisense transgene insertion. Fifteen plants were then determined as

transgenic AsTLP8 RNAi plants for the complete RNAi transgene amplification. It demonstrated a high transformation efficiency of 7.4% (Table 4). These are shown in Figure 19 lanes 2, 5, 7, 11, 14, 16, 20, 22, 23, 25, 27, 29, 30, 31, 34. These plants were then named as Rp 3.3, Rp 10.2, Rp 17.1, Rp 27.4, Rp 48, Rp 51.5, Rp 7.4, Rp 15.8, Rp 15.9, RP 15.13, Rp 55, Rp 64, Rp 84, Rp 81, and RP 91. Each of the T0 transgenic lines was planted with a minimum of 4 seeds each to generate T1 lines and investigate the heritability of the RNAi insertion.

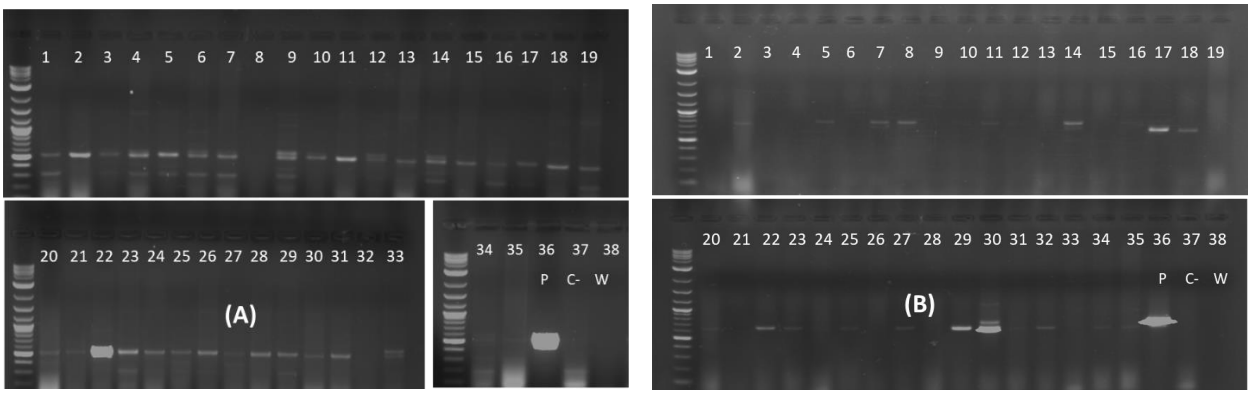

Figure 19. The molecular characterization in T0 Avena sativa transgenic plants. Lane number 1-35: putative transgenic plants, 36: Plasmid, 37: Negative control, and 38: water. A. The confirmation of sense RNAi insertion and B. The confirmation of antisense fragment insertion.

Table 4. The transformation record in Avena sativa bombarded with AsTLP8.pMCG161 construct

Construct	Number of calli	Putative transgenic Calli Frequency (%)	Transgenic plants in T0	Plant Transformation Efficiency (%)	Selection
AsTLP8. pMCG161	408	49.8% (203 of 408)	15	7.4% (15 of 408)	Bialaphos 3 mg/L (3 rounds)

3.4.7 Molecular Characterization in T1 Plants

After T0 plants having seeds, the T1 generation was advanced. One hundred and sixty-seven T1 plants were generated with two plants harbored the RNAi fragment in both insertions. It was proven by the PCR amplification at 802 bp using primer ZmAdh1.F1 & Os-waxy-AR.1 (sense fragment) and 775 bp using the primer pair of OsWaxy-A.F1 and OCS_Ter.R1 (antisense insertion). Besides, the intron flanked by both RNAi fragments was also checked using a couple of primer AF-7 and AF-9 to ensure the full RNAi transgene insertion. Both two plants displayed the amplification at 543 bp, as expected. Those plants were then named RP 17.1-3 and RP 84-18. It demonstrated a 1.19% integration rate of RNAi insertion fragment from T0 to T1 plants (Figure 20).

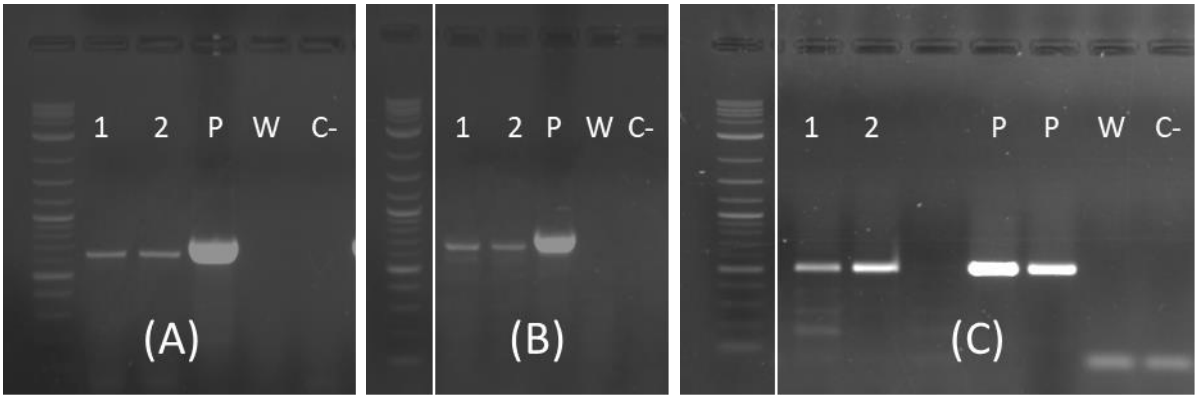

Figure 20. Molecular analysis of T1 RNAi transgenic plants. (A) displayed the amplification at the first RNAi insertion, (B) amplification at the second insertion, (C) amplification at the intron flanked by both fragments. Lane 1: plant RP 17.1-3, lane 2: RP 84-18, P: plasmid, W: water, C-: negative control.

3.4.8 Examination of T2 Transformants during Germination and Vegetative Stages

After T1 producing seeds, they were subjected to the Bialaphos 3 mg/L regeneration medium to check the *Bar* gene expression. Germination was observed and compared between putative transgenic seeds and non-transgenic seeds. Three out of 6 transgenic T1 seeds of Rp 17.1-3 (number 2) and 6 out of 6 seeds of Rp 84-18 (number 3) plants were survived and germinated, while none of the non-transgenic seeds (number 1) were germinated (Table 5)(Figure 21). It demonstrated that germinated T2 transgenic seeds inherited the *Bar* gene were resistant to Bialaphos herbicide.

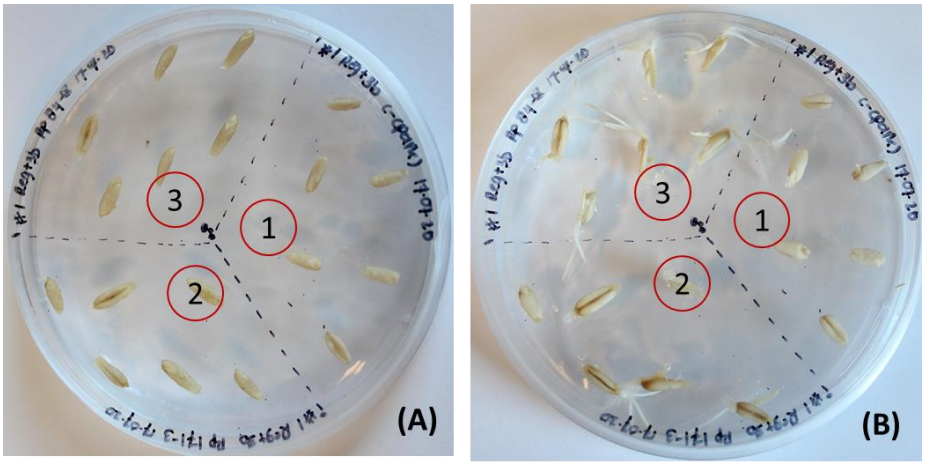

Figure 21. Seeds of transgenic plants Rp.17.1-3 (2), Rp 84-18 (3), and non-transgenic plant (1) under regeneration medium containing Bialaphos 3 mg/L. Figure A demonstrated seeds before imbibition; figure B showed seeds after 72 h of imbibition.

Table 5. The number of germinated seeds under the regeneration medium with Bialaphos 3 mg/L in 72 hours after imbibition

Plants	Number of Germinated seeds
Rp 17.1-3	4 0f 6 plants
Rp 84-18	6 0f 6 plants
Non transgenic plants	0 0f 6 plants

Furthermore, the basta painting was performed in T2 young leaf plants by applying commercial herbicide glufosinate liberty (0.2%). Putative transgenic plants exhibited substantially less herbicide symptoms than non-transgenic plants. A similar survival rate was observed in leaves under glufosinate herbicide (0.2%). All T2 plants from Rp 84-18 plants were healthy and green; whereas, only two out of six T2 RP 17.1-3 plants were resistant to the herbicide (Figure 22). Based on basta painting data and supported by germination rate, It indicated that RP 84-18 plants are homozygous, but RP 17.1-3 plants are still segregating and thus heterozygous that need further confirmation with a molecular approach.

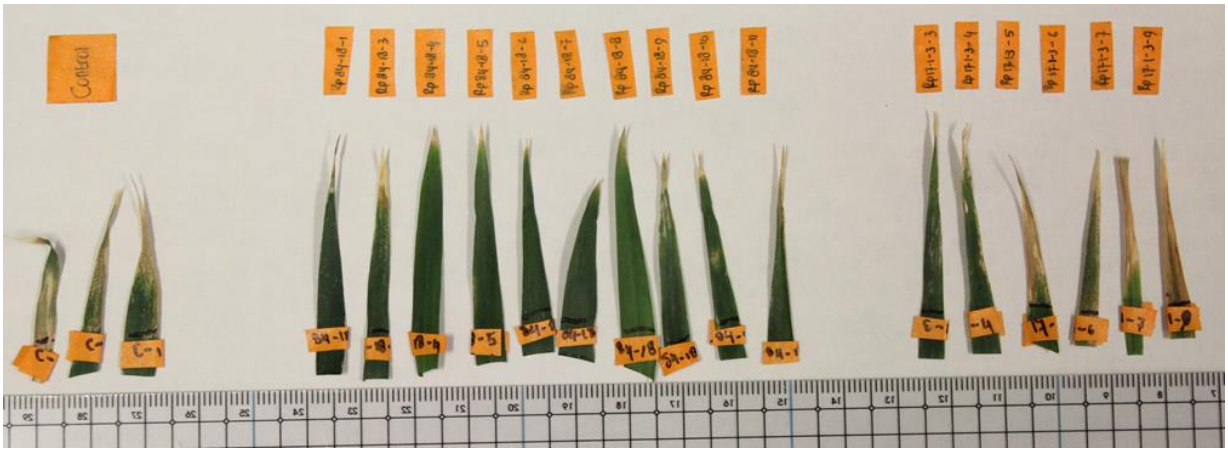

Figure 22. Basta leaf painting was performed in putative transgenic plants and non-transgenic plants in T2 generation under the application of 0.2% herbicide glufosinate liberty.

3.4.9 *TLP8* Expression in T2 Transgenic seeds

The *AsTLP8* expression was analyzed in different seed imbibition time (0h, 16h, 48h, and 96h) to identify the transcript abundance of *AsTLP8* in different germination stages. The expression was checked using the qRT-PCR technique with qAsTLP8F and qAsTLP8R primers (Table 6). In T2 dry seeds, expression of *AsTLP8* in transgenic transformants was significantly reduced by more than half time than non-transgenic seeds. However, during the germination stages, Rp 17.1-3 showed significantly higher than RP 84-18 and non-transgenic seeds that need further investigation (Figure 23). It is important to note that Rp 17.1-3 is not a homozygous line.

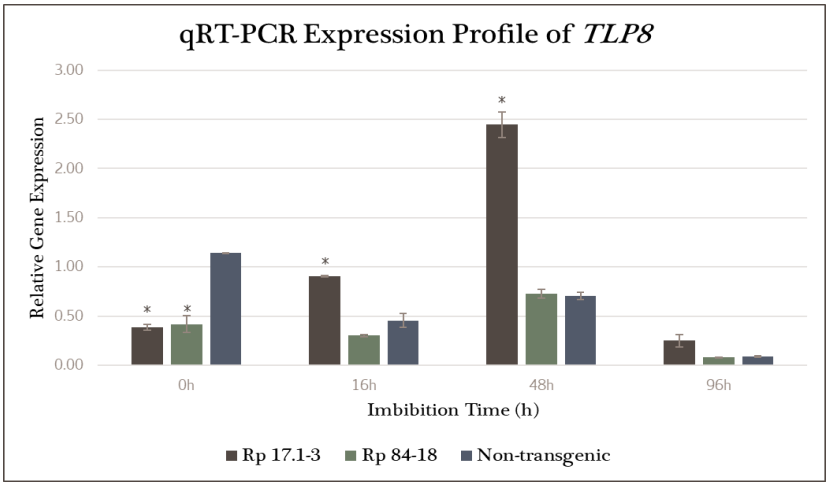

Figure 23. qRT-PCR expression abundance of AsTLP8 in T2 transgenic seeds (Rp 17.1-3, Rp 84-18) and non-transgenic seeds in different imbibition time (0h, 16h, 48h, and 96h).

This finding was also supported by RT-PCR analysis with AsTLP8F and AsTLP8R primer relative to *β-actin* gene expression (Figure 24). During germination, transgenic transformants RP 84-18 and RP 17.1-3 expressed more than non-transgenic seeds. It was also demonstrated that the highest *TLP8* expression was seen within 48h after imbibition.

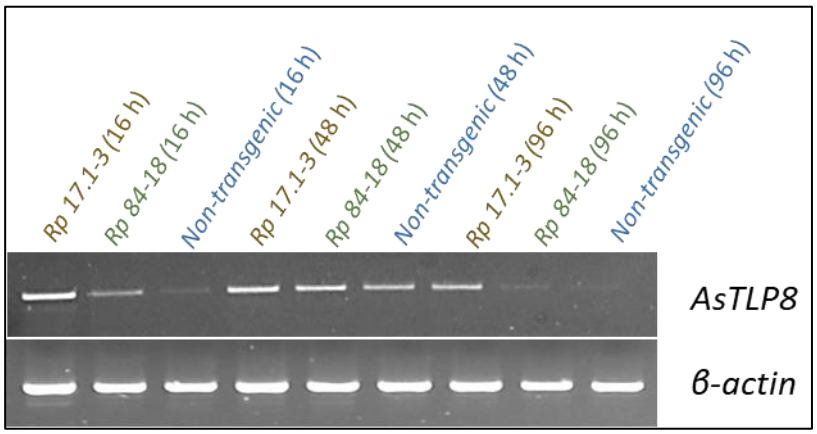

Figure 24. RT-PCR transcript expression of AsTLP8 in T2 transgenic seeds (Rp 17.1-3, Rp 84-18) and non-transgenic seeds during germination (16h, 48h, and 96h).

3.4.10 Comparison of Germination Growth Stages Between Transgenic and Non-transgenic Plants

The germination stages of transgenic plants and non-transgenic plants after 96 hours of imbibition was observed in the regeneration medium. Interestingly, both transgenic plants, Rp 17.1-3 and Rp 84-18 (Figure 25 A and B) germinated faster than non-transgenic plants (Figure 25 C). Transgenic seeds have emerged both shoot and root organ; nevertheless, non-transgenic seeds germinate only the shoot organ with a shorter size. These results are inconclusive considering the level of *TLP8* in different events, therefore warrant further investigation.

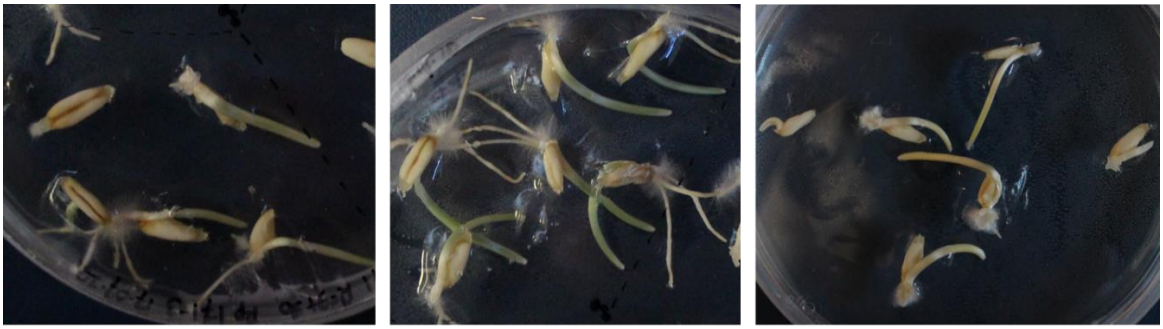

Figure 25. Germination stage of transgenic Rp 17.1-3 (A), RP 84-18 (B) lines in comparison to non-transgenic seeds (C).

3.5 Discussion

β-glucan is one of the significant components of cereals' endosperm cell walls (Burton & Fincher, 2014; Fincher & Stone, 1986). Within the endosperm cell wall of barley and oat, β-glucan accounts for 75% of the polysaccharide content, 20% is contributed by arabinoxylans, and 3 - 4% cellulose (Fincher & Stone, 1986). Multiple approaches have been conducted to identify genes associated with β-glucan biosynthesis. *Cellulose Synthase A* (*CESA*) and *Cellulose Synthase-Like* (*CSL*) gene families were reported to be involved in biosynthesis (Kaur *et al.*, 2017; Kaur *et al.*, 2016). However, these genes take only part of a total β-glucan content synthesis, as reported elsewhere (Newell *et al.*, 2012). Also, the overexpression of the *CSL* genes in *Arabidopsis thaliana*, which substitutes no β-glucan, resulted in a low level of β-glucan accumulation (Burton *et al.*, 2006; Doblin *et al.*, 2009). Therefore, it is suggested that other proteins might also play a role in β-glucan synthesis and regulation. Recently, the barley *Thaumatin-Like Protein 8* (*TLP8*) gene has been reported to associate malting quality in barley. Higher transcript of *TLP8* in malting barley genotypes deciphers lower β-glucan content and vice versa (Singh et al., 2017). Nevertheless, no report is published about the role of TLP8 function in oat β-glucan synthesis.

This research aimed to characterize *TLP8* in oat functionally. The previous report indicated that Thaumatin-Like Proteins acts like endo β-1,3-glucanase which hydrolyze 1,3 β-glucan, leading to increased membrane permeability (Osmond *et al.*, 2001). Furthermore, Singh et al. (2017) established that TLP8 interacts with 1,4 β-glucan, a key component of barley β-glucan moiety. Therefore, *TLP8* is an excellent candidate to be investigated further in another cereal, oat, which is now dominating the food industry due to β-glucan content. We decided to knock down this gene in oat variety, Park, as it is amenable to regeneration and transformation reported elsewhere (Cho *et al.*, 1999) and in our laboratory (Mahmoud, 2019).

A binary vector pMCG161 with a constitutive promoter was chosen due to its efficiency for silencing purposes reported in some monocots, such as maize, barley, and wheat (Bhati *et al.*, 2016; McGinnis *et al.*, 2005; Zalewski *et al.*, 2010). Rice waxy intron was used as a spacer between sense and antisense *TLP8* transgene sequence. It is reported that an intron's addition enhances silencing efficiency (Smith *et al.*, 2000). The intron-spliced hpRNA vector is used to stabilize the inverted repeat DNA by facilitating the sense and antisense sequences to find their random self-complementarity in the spliceosome complex to create duplex hpRNA formation (Wesley *et al.*, 2001). Thus, a non-spliced vector produces a lower steady-state of duplex hpRNA than a spliced one (Wesley *et al.*, 2001). This type of vectors has performed a significant silencing success in many organisms, for instance, in barley to target *CKX* genes activity (Zalewski *et al.*, 2010), wheat to target IPK1 genes activity (Aggarwal *et al.*, 2018), and to downregulate PsCPK1 expression (Qi *et al.*, 2018), and rice to target genes resistance to RSV virus (Li *et al.*, 2016).

As the tissue culture system and transformation of *Avena sativa* var. park was well established (Cho *et al.*, 1999; Somers *et al.*, 1996), it helped us create a successful transformation in our experiment. We selected the transformant under the selection of Bialaphos 3 mg/L herbicide glufosinate (liberty). It aimed to investigate the *Bialaphos Resistance* (*Bar*) gene expression harbored in the pMCG161 vector. Putative transgenic plants exhibited mild necrosis as compared to non-transgenic plants. It is explained that crops expressing the *Bar* gene could metabolize glufosinate herbicide by converting L-phosphinothricin into a non-phytotoxic metabolite of glufosinate, N-acetyl-L-phosphinothricin (Takano & Dayan, 2020); thus, the photosynthesis and photorespiration would not be agitated. *Bar* gene has been successfully applied in our transformation as a selectable marker as have been reported in rice (Nakamura *et al.*, 2010; Rathore *et al.*, 1993), maize (McGinnis *et al.*, 2005), sorghum (Grootboom *et al.*, 2010), barley (Tingay *et al.*, 1997), and wheat (Bhati *et al.*, 2016; Dong *et al.*, 2016; Vasil *et al.*, 1992)

Out of 203 plants generated, fifteen plants carried the AsTLP8 RNAi transgene in T0 generation, indicating a relatively high transformation efficiency (7.4%). The efficiency is influenced by many factors, including the type of silencing cassette, targeted gene, and the type of plant species (Travella *et al.*, 2006). Despite having a high transgenic frequency at the T0 stage, only two independent plants out of 176 plants carried the mutation in the T1 generation. Reduced transmission of RNAi transgenes has also been reported in maize

(McGinnis *et al.*, 2005). Even though the independent RNAi transgenic line is low, the selection *Bar* gene is carried forward with high efficiency. About 65% of T1 plants were resistant to the application of glufosinate herbicide shown in Figure 18. Similar events were also observed by McGinnis *et al.* (2005).

As expected, this study showed that hpRNA-mediated silencing could drive efficient sequence-specific interference. The transcript level of *AsTLP8* was significantly reduced to about 2.5-fold in T2 dry transgenic seeds compared to non-transgenic seeds and made our RNAi experiment successful. It could be explained that the AsTLP8 RNAi transgene complementing with endogenous *AsTLP8* transcripts leading them to degradation; thus, the lower expression was observed. Similar phenomena were observed in *Arabidopsis thaliana* for *Pan* gene (Chuang & Meyerowitz, 2000), wheat for *γ-gliadins* gene (Gil-Humanes *et al.*, 2008), barley for *HvCKX* gene (Zalewski *et al.*, 2012), and maize for six targeted genes (McGinnis *et al.*, 2005).

Interestingly, *AsTLP8* RNAi transgenic plants expressed differently during germination. Transgenic plants RP 17.1-3 have a significantly higher *TLP8* expression in 16h and 48h compared to Rp 84-18 and non-transgenic plants, which share about the same expression level. A possible explanation could be either the methylation level changes (Wojtasik et al., 2014) or incorporated additional copies with partial fragments of *AsTLP8*, which needs further investigation. During particle gene gun bombardment, such breakages and events are not unusual and reported in some organisms, such as maize (McGinnis *et al.*, 2005), Barley (Zalewski *et al.*, 2012), and oat (Pawlowski & Somers, 1998; Svitashev *et al.*, 2002), which could either silence or activate the genes expression. Thus, the rearranged genomic sequence probably produced during the particle gene gun bombardment could be a reason for the *TLP8* overexpression.

In terms of methylation level, double-stranded RNA can recruit methyltransferase involved in the DNA methylation process, consequently modulates gene expression (López *et al.*, 2011). The change either cause suppression or activation in particular genes (Chinnusamy & Zhu, 2009). Therefore, the methylation level change could also be an origin of *TLP8* activation upon the treatment.

It was observed that a higher *AsTLP8* expression was correlated to a faster germination growth, as expressed by the expedited emergence of shoot and root and development of these organs. Conversely, a low level of *TLP8* indicates a late growth of shoot and root in non-

transgenic plants. Based on our investigation, it is suggested a probable function of TLP8 during oat germination stages. The TLP8 may bind to β-glucan, leading to a weakening of the cell wall and breaking the dormancy (Singh *et al.*, 2017). After the cell wall breakage, seeds start to absorb the water and activating the enzyme, which consequently induces the development of shoots and roots; thus, germination starts (Bentsink & Koornneef, 2008). It is supported by the examination in the Morex-Genes barley RNA sequencing database. A significant *TLP8* expression was detected during embryo development (Iqbal *et al.*, 2020). However, questions remain about the expression of AsTLP8 and the characterization of transgenic events for their association with β-glucan content during the germination process. To conclude, the RNAi transformants developed in this study are important mutants to further experiments for investigating the role of *TLP8* in the modulation of oat β-glucan.

3.6 Conclusion

TLP8 gene ortholog was retrieved in oat, and the RNAi construct has been successfully created. Using particle gene gun bombardment, transgene RNAi was introduced, and transformants were subjected to herbicide bialaphos (3mg/L) and glufosinate liberty (0.2%) to select the putative transgenic ones. Our experiment successfully developed a high putative transgenic calli frequency of 49.8% and plant transformation efficiency of 7.4% in the T0 generation. Two transgenic transformants were generated in the T1 generation, named RP 17.1-3 and RP 84-18. As expected, a significant reduction of *TLP8* transcript was observed in T2 dry seeds in both transformants compared to non-transgenic seeds.

Interestingly, an inverse phenomenon was also observed where *TLP8* expression increased remarkably during the germination stage. A high expression correlated with a faster germination growth, which needs further investigation. To conclude, our experiment has provided TLP8-knockdown transformants as useful resources for subsequent analysis of TLP8 function and correlation with β-glucan biosynthesis and regulation.

CONNECTING STATEMENT BETWEEN CHAPTER III & IV

The comprehensive understanding of β-glucan biosynthesis is limited, and further knowledge is required, which we were unable to obtain in Chapter III. The fact that other complex genetic factors are involved in the biosynthesis of β-glucan. Therefore, another alternative approach, utilizing the maize *Ac*/*Ds* mediated mutagenesis, has been applied for the creation of genetic resources for the future identification of additional genes.

Diploid *Avena strigosa* was chosen for this study to overcome the redundancy problem in hexaploid oat. Besides, *Avena strigosa* is considered a high β-glucan oat with composition is about 2.85-6.23% (Redaelli *et al.*, 2013). It is also supported by the fact that the diploid As genome has been successfully sequenced and annotated (Maughan Peter *et al.*, 2019).

In this chapter IV, the experimental design of *A strigosa* transformation and transposon mutagenesis, including a list of various *Ac*/*Ds* constructs and molecular and histochemical assays in transgenics plants, have been documented.

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

CHAPTER IV: Standardizing the Tissue Culture and Transformation System and Developing Transposon Functional Genomic Resources in Diploid Oat

4.1 Abstract

We introduced the maize *Ac* and *Ds* transposable elements into the diploid *Avena strigosa* oat genome to create a transposon-mediated functional genomic resource in oat, especially to tag genes associated with β-glucan. Different *Ac/Ds* constructs and strategies were applied. The *Ac/Ds* elements were introduced into the embryogenic region of high β-glucan diploid (*Avena strigosa*) oat by particle gen gun bombardment. Transformants were generated and selected using a *Bialaphos* (*Bar*) and hygromycin (*Hpt*) selectable marker genes, which yielded up to 42.8% putative transgenic calli frequency and 66.6% transformation efficiency. Histochemical assays confirmed the expression of *Bar,* and transgenic plants were found to be resistant to glufosinate herbicide LIBERTY (0.2%). Molecular characterization confirmed that three plants harbored *Ds* insertion, 11 plants carried *Ac* insertion, and two plants had double *Ac* and *Ds* insertion. These independent transgenic lines provide essential donor plants for subsequent functional genomic studies. The transposon activity was examined transiently by co-transforming *Ac* and *Ds*- interrupted *Gus* constructs and advancing the stable transgenic events possessing both *Ac* and *Ds* elements. *Ds* excisions were identified in transgenic T1 plants harboring *Ac* construct and *Ds* activation tagging constructs. Data was supported by molecular PCR characterization and histochemical Gus assays. No difference was observed using *Bar* or *Hpt* selectable marker genes, indicating that both are good selective agents in *Avena strigosa* transformation. In conclusion, our *Ac*/*Ds* system worked efficiently and effectively in generating both a transient and stable transformation system in diploid oat, making it a valuable tool for further gene tagging and functional approach in oat.

4.2 Introduction

Diploid sand oat (*Avena strigosa*) is one of four cultivated oat species besides *Avena sativa*, *Avena byzantina*, and *Avena abyssinica*, which have been grown as winter season cover crop, cereals, and feeding livestock (Kuszewska & Korniak, 2009; Podyma *et al.*, 2019; Suttie & Reynolds, 2004). *A. strigosa* grows in a wide range of landscapes, even in the least fertile fields, and have high resistance against pathogens and diseases (Kuszewska & Korniak, 2009; Rayapati *et al.*, 1994; Steinberg *et al.*, 2005). *A. strigosa* also contain essential health-promoting compounds, including β-glucan, tocopherols, tocotrienols, and AVAs (Smitterberg,

2018). Compared to most commercial oat, *A. sativa*, *A. strigosa* has a higher β-glucan content, about up to 6.23% (Redaelli *et al.*, 2013; Welch *et al.*, 2000). Furthermore, it has been reported that *A. strigosa* contains 27-52% more proteins, 14-27% more fats, and 38-72% more sugars than the common oat (Kuszewska & Korniak, 2009). Although *A. strigosa* provides essential nutritive compounds, genetic resources in this species are not extensively exploited and identified (Podyma *et al.*, 2019). Therefore, this species deserves further attention since it provides significant genetic variability for health-promoting traits.

Various functional genomic approaches have been used to explore and characterize genes in the plant genome, such as T-DNA insertion, RNA interference, and transposons tagging tool (Alonso & Ecker, 2006; Li *et al.*, 2011; Singh *et al.*, 2012). The transposon-based insertional mutagenesis approach has advantages over other tools. The system is relatively fast and straightforward as it does not require transformation for every single gene investigation (Singh et al., 2006). Once the transposon elements are successfully integrated, these could help generate large mutant populations by crossing, reducing the need for complicated and time-consuming tissue culture and transformation steps (Singh *et al.*, 2006; Wu *et al.*, 2019). Many transposable elements have been identified, and maize *Ac*/*Ds* elements are one of those that has been characterized in detail. The *Ac* elements encode 807 amino acid transposase enzymes, inducing the transposition activity when recognizing the 11-bp Terminal Inverted Repeat (TIR) (Du *et al.*, 2011; Lazarow & Lütticke, 2009). The *Ds* element, a mutated *Ac* element, lacks a transposase coding region while retaining TIRs and subterminal regions of 250-300 nucleotide (Singh *et al.*, 2006). It depends on the *Ac* elements for activation and transposition (McClintock, 1951). The use of transposable elements for functional gene identification was first reported in tobacco (Baker *et al.*, 1986). Since then, *Ac*/*Ds* mediated mutagenesis has been successfully utilized in many crop species, for instance, rice (Jiang *et al.*, 2007), Arabidopsis (Fridborg *et al.*, 1999; Wilson *et al.*, 1996), wheat (Takumi, 1996), and barley (Ayliffe *et al.*, 2007; Koprek *et al.*, 2000).

Understanding the structural features of the Ac/Ds sequence leads researchers to reorganize and reconstruct the elements for different functional genomic purposes, either for gene knockout or for gene activation. Gene knockout occurred when the *Ac*/*Ds* elements landed in the genic area, causing the sequence reorganization (Jiang *et al.*, 2007). Such an experiment has been demonstrated by Jiang *et al.* (2007), introducing *Ac/Ds* to disrupt rice's endogenous genes. Due to its disruption, they successfully created rice mutants with a higher yield and

responsive to abiotic stresses. Whereas, gene activation could be developed when the construct containing enhancer or promoter sequence is integrated into Untranslated 5' region adjacent to particular genes, inducing the gene's expression (Ayliffe *et al.*, 2007; Jiang *et al.*, 2007). Due to these advantages, supported by the tendency of *Ac/Ds* elements to transpose to a linked region (Koprek *et al.*, 2000; Osborne *et al.*, 1991; Singh *et al.*, 2012), maize *Ac/Ds* transposon tagging approach has been considered a powerful tool for functional genomics studies.

Previously, Mahmoud (2019) has successfully utilized the maize *Ac/Ds* tagging approach for gene exploration and identification in common oat *A. sativa,* a first successful report of transposon tagging in the oat genome. Several sequences have been successfully cloned and identified; one of them was homologous with barley *Gibberellic Acid 20 oxidase 3 (GA20ox3)* gene (Mahmoud, 2019). However, there has not been a report of the maize *Ac/Ds* system application in the diploid *A. strigosa* genome. Therefore, introducing the *Ac/Ds* element in *A. strigosa* would allow us to explore the gene pool and reveal beneficial genes of *A. strigosa*.

Based on the successful introduction of the maize Ac/Ds elements in the common oat genome, we made efforts to insert Ac*/Ds* elements in diploid *A. strigosa* to develop genetic resources for functional characterization and identification of unique genes. To support that, we have also standardized the tissue culture and transformation system for the first time in the diploid *Avena strigosa*.

4.3 Materials and Method

4.3.1 Sample Preparation and Tissue Culture Induction

Mature seeds of diploid (*Avena strigosa*) were surface sterilized in 20% bleach (5.25% sodium hypochlorite) for 20 minutes, followed by 3-4 rinses in sterile distilled water. The sterilized seeds were subjected to a regeneration medium, which is an MS-based medium (Murashige & Skoog, 1962), supplemented with sucrose 30 mg/L, thiamine HCL 1 mg/L, pyridoxine HCL 0.5 mg/L, nicotine acid 0.5 mg/L, CuSO4 0.16 mg/L, Indoleacetic acid (IAA) 1 mg/L, 6-benzyl amino purine (BAP) 1 mg/L hormone, and solidified Gelzan 2.8 g/L (Table 3). The medium was set to pH 5.8 and autoclaved at 121^0C for 20 minutes. The plates were incubated for 3-4 days in 16 hours light and 8 hours dark system. Germinated shoot and root were manually removed. The embryogenic part was then placed in a callus induction medium (DBC3), a second-step of calli induction medium, consisted of MS basal medium, maltose 30 g/L, casein

1g/L, myo-inositol 0.25 g/L, proline 0.69 g/L, thiamine HCL 1 mg/L, $CuSO_4$ 5 µM, and the combination of 2.4-dichlorophenoxyacetic acid (2.4-D) 1 mg/L and BAP 0.5 mg/L (Table 3). The samples were then incubated for two weeks to generate green calli. To maintain it, calli were subcultured every two weeks in DB3 induction medium.

4.3.2 Particle Gen Gun Bombardment

The Bio-Rad Biolistics PDS-1000/He was used to bombarding several Ac/Ds constructs into green calli of *Avena strigosa*. Calli were firstly subjected to an osmotic medium for 4 hours before the bombardment and 24 hours after that. It aimed to increase the DNA uptake, thus will increase the transformation efficiency (Cardinal et al., 2016). The osmotic medium is a callus induction medium (DBC3) without maltose and a 0.2 mannitol and a 0.2 sorbitol (Table 3). The calli bombarded were placed in a 16h light and 8h dark system at 25^0 C (Able et al., 2001). For the transformation, the methodology developed by Cardinal *et al.* (2016) and Kikkert *et al.* (2005) was followed. 12 µg/L DNA of each construct was used for six plates. The DNA-coated gold microcarriers were prepared prior to the shooting. Firstly, 36 µl of gold suspension was taken into a new tube and followed by centrifugation for 30 seconds at high speed. The supernatant was then removed, and 200-300 µl of water was added slowly. It was followed by centrifugation for 3 minutes at 2000 rpm. The plasmid DNA was then added into the tube and gently tapped. Sterile water was added to complete 250 µl with the DNA. The other solution, 250 µl $CaCl_2$ and 50 µl (0.1M) spermidine, were added. The solution was mixed by tapping, followed by vortex, which then kept on ice for 30 minutes with mixing every 10 minutes. It was then centrifuged for 5 minutes at high speed. The supernatant was removed. It was then washed by 200-300 µl ethanol absolute followed by centrifugation for 1-2 minutes with high speed. After removing supernatant, the 36-60 µl of ethanol absolute was added. Six µl of precipitation solution was dispensed into the microcarrier disc and let to dry for 5-10 minutes. The bombardment was performed using an 1100 psi rupture disk (Bio-Rad). The vacuum was switched to 25-28 mm Hg, and target calli were bombarded.

4.3.3 *Ac/Ds* Plasmid Construct

In the experiments, several *Ac/Ds* constructs have been used for transformation. The detail of each construct is explained below.

1. pSPWDV-ActIDsUbibarDsGus (McElroy *et al.*, 1997).

This plasmid contains pSP27 (pSP, Promega) as the backbone, a replication-associated region from the wheat dwarf virus (WDV), and the replication-associated protein's coding region

(Rep). The rice Actin1 (*Act1*) 5' region consists of *Act1* promoter, first non-translated exon, and first intron. The maize *Ds* elements are cloned from pDS7 (Koprek et al., 2000), composed of 254 bp of Ds5' sequence and 320 bp of Ds3' sequence. The maize ubiquitin (*Ubi*) region contains the *Ubi* promoter, first non-translated exon, and first intron and followed by the *Bar* gene from the *Streptomyces hygroscopicus* phosphinothricin acetyl transferase. The *Gus* gene consists of the β-glucuronidase coding region of *E. coli*. While the 3' transcription terminator region is derived from the *nopaline synthase* (*nos*) gene of *Agrobacterium tumefaciens* (Figure 26).

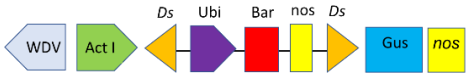

Figure 26. Schematic representation of pSPWDV-ActIDsUbibarDsGus construct

2. pSP-DsUbibar (Koprek *et al.*, 2000).

The construct uses pSP plasmid as the backbone, consisting of the selectable *Bar* gene under Maize *Ubi* promoter and first intron. The *Bar* gene is flanked by 254bp of *Ds*5' sequence and 320 bp of *Ds*3' sequence having an *Actpase* recognition site (Figure 27).

Figure 27. Schematic representation of pSP-DsUbibar construct

3. pCambia-UbiAcnos.

This construct contains the transposase coding sequence under the regulation of maize *Ubi* promoter and *nos* transcription termination (Figure 28). The construct harbors a transposase enzyme to induce transposition activity.

Figure 28. Schematic representation of pCambia-UbiAcnos construct

4. pWBVec8-DsUbiBarUbiDsGUS (Ayliffe *et al.*, 2007).

The construct was generously provided by Dr. Michael A. Ayliffe, CSIRO Plant Industry, Canberra, Australia. The construct contains 250 bp 5' and 3' ends of the maize *Ac* transposable elements (*Ac wx-m9*, GenBank K01964) along with an internal non-transcribed 909 bp Transposase coding sequence. The strong *Ubi* promoter at each *Ds* end is included for the activation tagging. A *Bar* selectable gene is under the regulation of a CaMV 35S promoter and Agrobacterium OCS transcriptional termination sequence. Adjacent to the 3' *Ds* sequence, a promoterless G*us* gene with *nos* transcription termination sequence has been included (Figure 29).

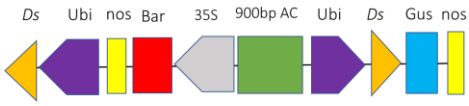

Figure 29. Schematic representation of pWBVec8-DsUbiBarUbiDsGUS construct

5. pAct1HPT-4 (Cho *et al.*, 1998).

Plasmid pAct1HPT-4 possesses hygromycin antibiotic selection. The *Hygromycin B Phosphotransferase gene* (*Hpt*) from *Escherichia coli* is driven by *Act1* promoter and *nos* terminator (Figure 30).

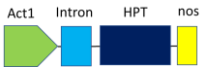

Figure 30. Schematic representation of pAct1HPT-4 construct

6. pAHC20 (Christensen & Quail, 1996).

Plasmid pAHC20 consists of the Bar gene driven by maize *Ubi1* promoter and contains 5' 83bp untranslated exon, and the first intron (1010bp). This was used in co-bombardment in order to select putative transgenic calli under Bialaphos and Glufosinate herbicide treatment. The transcription is terminated by *nos* transcriptional terminator sequence (Figure 31).

Figure 31. Schematic representation of pAHC20 construct

4.3.4 Transient Gus Expression

Gus assay was performed to test β-glucuronidase activity following the staining method developed by Jefferson *et al.* (1987). The GUS assay solution was composed of 3.79 ml of sterile deionized water, 200 µl of 0.5M EDTA, 10 µl of 0.1% Triton X-100, 500 µl of 10 mM K_4 ferrocyanide ($K_4Fe(CN)_6.3H_2O$), 500 µl of 10 mM K_3 ferricyanide ($K_3Fe(CN)_6$), 5 ml of phosphate buffer, and 5 mg of X-Gluc which had been diluted in dimethyl sulfoxide (DMSO) solution. The samples (calli, seeds, and leaves) were incubated in Gus assay solution pH 7.0 at 37°C. For green samples, they were incubated into acetone before its treatment with the GUS solution. A transient assay for *Ac/Ds* bombardment was performed on the green and healthy *A. strigosa* calli. Calli were bombarded and co-bombarded with pSPWDV-ActIDsUbibarDsGus construct, which *Ds* elements interrupted *β-glucuronidase* (*Gus)* gene expression and pCambia-UbiAcnos construct that provided a transposase enzyme to catalyze the transposition events. Calli were subjected to the osmotic medium 4 hours prior to bombardment. The histochemical gus assay analysis was performed 24 hours after bombardment to check the *Gus* gene expression. The assays have been optimized,

demonstrating that the assay was sensitive towards the imbibition time. The solution with a concentration of 0.05 M and 0.01 M are sufficient for the assay.

4.3.5 Selection of Putative Transgenic Calli

The calli were incubated overnight in 16 hours light and 8 hours dark system in the osmotic medium after bombardment. The calli were then sub-cultured into the selection medium. Calli transformed with construct harboring a *Bar* gene were sub-cultured in DBC3 medium with 3 mg/L of Bialaphos (IGNITE, Bayer Crop, Canada). Moreover, plants bombarded with a *Hpt* gene were subjected to DBC3 containing Hygromycin B (Sigma) 10 mg/L. Bombarded tissue was incubated for two weeks in the first selection medium plate. The survived green calli were sub-cultured into the second selection plate for the other one or two rounds, while the dead calli were discarded.

4.3.6 Regeneration of Plants

After the calli selection, the healthy calli were sub-cultured into regeneration medium followed by rooting medium (Table 3). Explants that successfully generated shoots and roots were moved into the soil.

4.3.7 Basta Leaf Painting

The basta painting was performed by applying commercial herbicide glufosinate liberty (0.2%) (IGNITE, Bayer Crop, Canada) in young leaf plants. The examination was conducted one week after the assay. Plants with green and healthy leaves were considered as putative transgenic plants conferring the *Bar* gene.

4.3.8 Genomic DNA Extraction

The DNA was extracted from young leaf plants using the urea extraction method (Chen & Dellaporta, 1994). The samples were collected and quickly transferred to liquid nitrogen for immediate extraction. Samples can also be stored at -80^{o}C for further use. The leaf was ground using Tissue Lyser 2 Qiagen Apparatus to gain a fine powder. 0.5 ml of urea buffer containing 5M NaCl, 2M Tris-HCL pH 8.0, 0.5 EDTA pH 8.0, and sarkosyl (N-laurylsarcosine) was added and mixed well. 0.5 ml phenol: chloroform (1:1) were added and mixed on a tabletop shaker for 1 hour. After the incubation, samples were centrifuged for 6 minutes. The supernatant was moved into a new tube and added with 1/10 vol 4.4M NH_4Ac and an equal volume of isopropanol. The tube was then inverted several times to precipitate the genomic DNA. The samples were centrifugated for two minutes, followed by a washing step with ethanol 70% and 90%. Pellet was then resuspended in 50 µl TE.

4.3.9 Molecular Characterization

The different sets of primers were used to detect *Ac* and *Ds* elements in the putative transgenic plants (Table 6). The PCR reaction was comprised of 10 µl master mix (GoTaq® Green Master Mix), 7 µl of molecular water, 1 µl of DNA sample, and each 1 µl 10uM for forward and reverse primer. The PCR condition was 94^0C in 30 seconds for denaturation, 30 seconds for annealing in a particular temperature based on the primer pair, and 72^0C for elongation in 1 minute.

Table 6. The list of primers used in the experiment

Primers	Orientation	Sequence	Detection	Size (bp)
AsActinF	Forward	GAGACCTTCAATGTTCCAGCCATG	*Actin* gene	700 bp
AsActinR	Reverse	ATACTTCCTCTCGGGCGGTG		
QnosF	Forward	AATCCTGTTGCCGGTCTT	*Ds* 3’	473 bp
QDs3R	Reverse	CGTACCGACCGTTATCGTAT		
DS5F	Forward	AGATAGGGATGAAAACGGTCG	*Ds* 5’	490 bp
Ubi.Int5R	Reverse	GATAAACTGCACTTCAAACAAGTGT		
AC3	Forward	ACCACCAGCACTGAACGCAGACTC	*Ac* element	852 bp
AC5	Reverse	AACCTATTTGATGTTGAGGGATGC		
HPTF	Forward	AAGCCTGAACTCACCGCGACG	*Hpt* gene	1000 bp
HPTR	Reverse	AAGACCAATGCGGAGCATATAC		
RNAi.BarF	Forward	TCAGATCTCGGTGACGGGC	*Bar* gene	799 bp
Ubi.blpR	Reverse	GGCATATGCAGCATCTATTCATATGC		
AC5	Forward	AACCTATTTGATGTTGAGGGATGC	Vec8	1000 bp
Ubi.intr5R	Reverse	GATAAACTGCACTTCAAACAAGTGT		
ubiDs-uidAF2	Forward	GGTTGGGCGGTCGTTCATTC	Transposition event of vec8	500 bp
ubiDs-uidAR2	Reverse	GCGGGATAGTCTGCCAGTTC		
OsWaxy-A.F1	Forward	CATTCATCTGATCTGCTCAAAGC	Rice waxy intron & OCS terminator	775 bp
OCS_Ter.R1	Reverse	ATCTGAGCTACACATGCTCAGG		
As_TLP8rsF	Forward	CGGACCGACTAGTCCGGCGGGGGCC	RNAi region	304 bp
As_TLP8rsR	Reverse	CCTAGGGAGCTCCGGGCAGGAAGTCCATGG		
ZmAdh1.F1	Forward	TTGCAAGGAGGCGTTTCT	Maize Adh and rice waxy intron	802 bp
OsWaxy-A.R1	Reverse	ACCATCTTGTGGAGCTAGTTG		
AF-2	Forward	GGCTGAACACATCATACGATATTGAGC	Maize Adh & RNAi region	532 bp
AF-3	Reverse	GCCCGAACTCGGCCAG		
AF-7	Forward	GGGGTTTTCCAACTAGCTCC	Rice waxy intron	543 bp
AF-9	Reverse	CCCGGAGATGCACAGAGC		
AF2	Forward	GGCTGAACACATCATACGATATTGAGC	Maize Adh & RNAi region	622 bp
As_TLP8rsR	Reverse	CCTAGGGAGCTCCGGGCAGGAAGTCCATGG		

AF7	Forward	GGGGTTTTCCAACTAGCTCC	Rice waxy intron & RNAi region	1167 bp
AsTLP8NR	Reverse	CTAGTCCGGCGGGGGCCA		
AfTBP2F	Forward	TGAGCCAGAACTTTTTCCTG	*TBP* reference gene	100bp
AfTBP2R	Reverse	CTTTGCACCGGTCAAGACA		
qAsTLP8F	Forward	CTACTCCAAGTTCTTCAAGGG	TLP8	100bp
qAsTLP8R	Reverse	GAAGATGACCTGATAGTTGGTG		

4.3.10 Development of *Ac/Ds* Oat line

The putative *Ac*/*Ds* transgenic plants were grown under the greenhouse condition; 24^0 C for day and 18^0 C for night temperature. The light provided 1000 µE light intensity and a 16:8 hours photoperiod. The fertilizer containing 20:20:20 (NPK) was applied one time per week to suffice the plant's nutrition.

4.4 Results

4.4.1 Tissue Culture and Transformation System in Diploid Oat

The samples used for tissue culture induction were diploid oat (*Avena strigosa*) (Figure 32A). After the induction in the DBC3 medium, the calli were formed, as shown in Figure 32B. The green and healthy calli were chosen for a further subculture to maintain the calli stock or directly used for bombardment. The selection was performed to select the putative transgenic calli conferring the selectable marker resistance gene (Figure 32C). The putative calli harboring the resistance gene survived under the herbicide or antibiotic exposure, while the non-transgenic calli died. The survived calli were good candidates for the regeneration and moved to the regeneration medium. When the shoot was growing well, as shown in Figure 32D, they were moved on to the rooting medium (Figure 32E), followed by transferring to the soil after developing enough healthy roots (Figure 32F). The tissue culture and transformation were applied to diploid oat and showed successful results due to the putative transgenic plants being successfully grown up to their whole life cycle (Figure 32A-H).

The transformation record is provided in Table 7, which includes the different constructs, number of calli, plants regenerated, regeneration frequency, transformation efficiency, and selection medium. The selection frequency was counted as the number of survived calli after selection. While the transformation efficiency showed the number of positive transgenic plants per total number of regenerated plants. In our experiment, significantly higher selection frequency (4.6% - 42.8%) at calli level and transformation efficiency (13.0% - 66.6%) from calli to putative transgenic plants was achieved in this diploid oat species. In addition,

both bialaphos and hygromycin were useful in the selection and identification of putative transgenic plants.

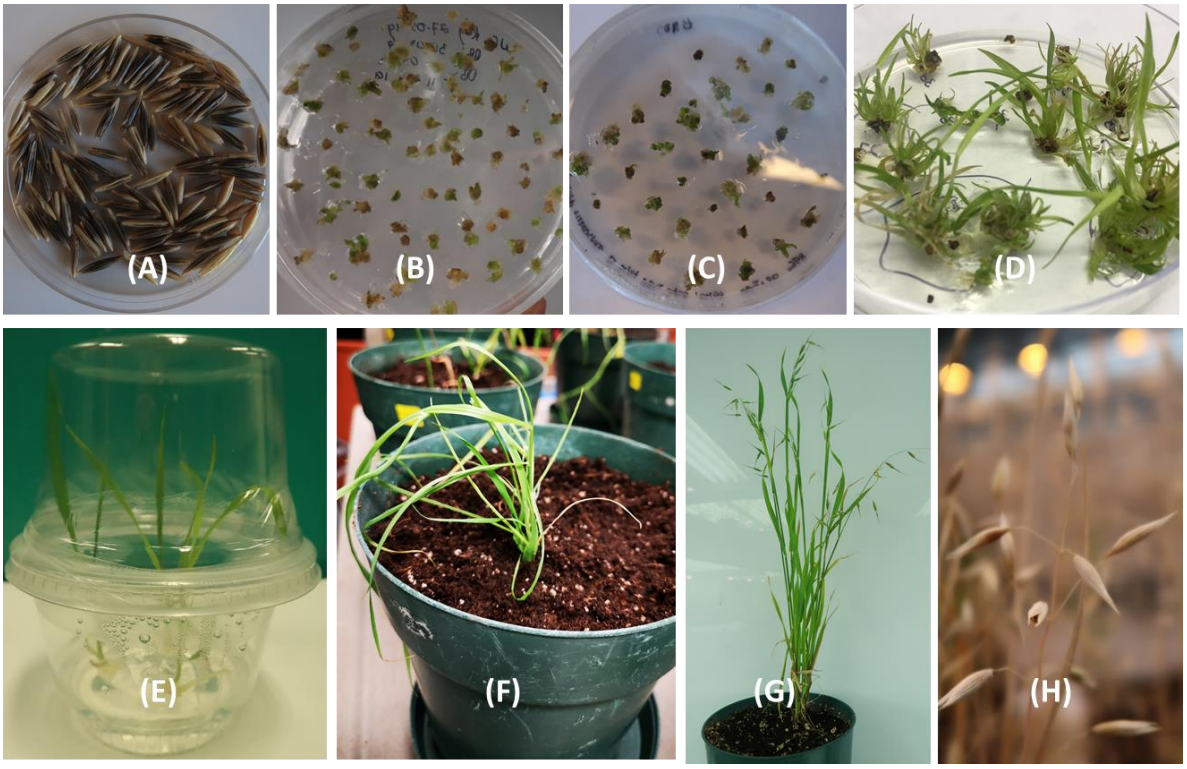

Figure 32. The diploid oat (Avena strigosa) tissue culture and transformation system. A. Diploid oat's seeds, B. Calli formed after induction in DBC3, C. the calli after the selection, D. The shoot was formed under regeneration medium, E. Plants in the rooting medium, F. Plants transferred into soil, G. Putative transgenic plants in the regenerative stage, H. Mature plants and ready to be harvested

4.4.2 Transient Expression Assay of Maize *Ac/Ds* System in Diploid Oat Genome

In calli, where both pCambia-UbiAcnos and pSPWDV-ActIDsUbibarDsGus constructs were successfully integrated, the transposase enzyme induced the *Ds* elements excised out of the pSPWDV-ActIDsUbibarDsGus, leading to the *Gus* gene expression (Cardinal *et al.*, 2016). The blue color was seen in calli bombarded with both constructs (Figure 33 A); nevertheless, calli with only pSPWDV-ActIDsUbibarDsGus vector did not express it because the *Ds* element was interrupting *Gus* expression (Figure 33 B). The transient assay confirmed that Maize *Ac*/*Ds* system worked in *A. strigosa* genome.

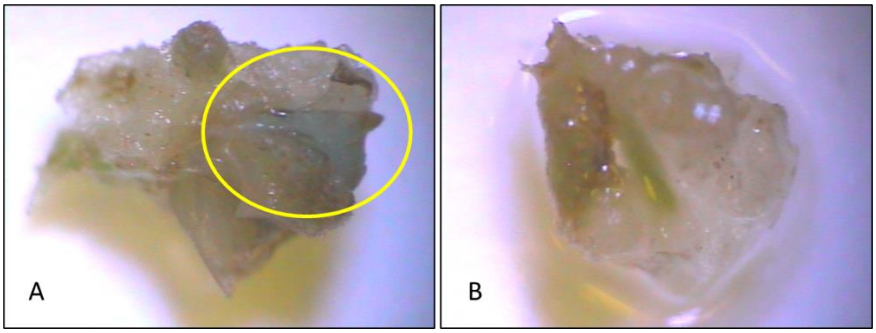

Figure 33. The expression of Gus gene assay; Figure A. Demonstrated the expression of Gus gene of the calli with pCambia-UbiAcnos and pSPWDV-ActIDsUbibarDsGus co-bombardment, while figure B. Displayed non expressed blue color transformed wit only pSPWDV-ActIDsUbibarDsGus construct

4.4.3 The transformation of oats with *Ac*/*Ds* Constructs

4.4.3.1 Selection of Putative Transgenic Transformants

Putative transgenic calli conferring the *Bar* gene revealed that most of the calli were survived under the exposure to bialaphos herbicide 3 mg/L (Figure 34A). The calli survival rate varied depending on the constructs used. Calli transformed with pSPWDV-ActIDsUbibarDsGus, pSP-DsUbibar, pAHC20 & pCambia-UbiAcnos, and pWBVec8-DsUbiBarUbiDsGUS & pCambia-UbiAcnos had 23 of 62 (37.1%), 7 of 60 (11.6%), 9 of 64 (14.1%), and 3 of 65 (4.6%) survived calli, respectively (Table 7). Likewise, putative transgenic plants exhibited substantially less herbicide symptoms (mild necrosis) than in control plants when performed with the commercial herbicide glufosinate liberty (0.2%) (Figure 34 B). It was concluded that calli and plants possessing the *Bar* gene were resistant to the application of bialaphos and glufosinate herbicide.

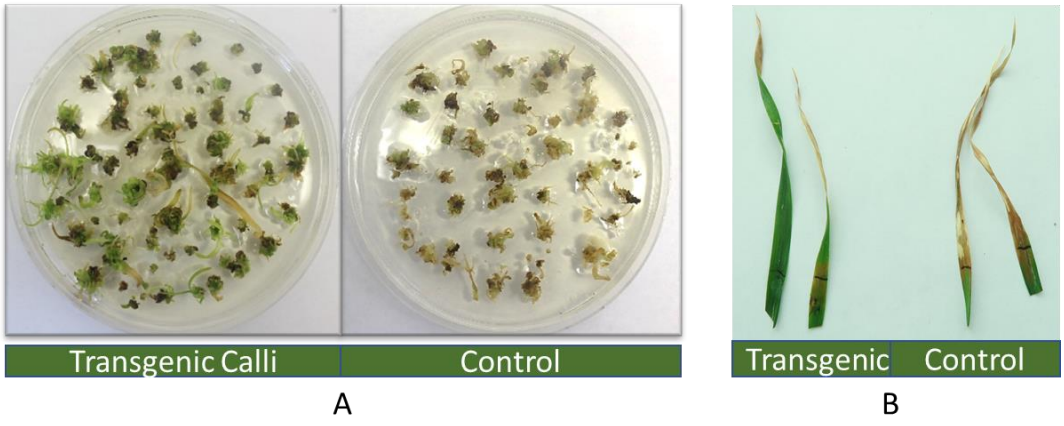

Figure 34. The selection of calli and plants conferred the bar gene under the selected herbicide. (A) The comparation of transgenic and non-transgenic calli under 3mg/L Bialaphos and (B) Leaf basta painting assay using 0.2% glufosinate liberty.

Furthermore, putative transgenic calli conferring the *Hpt* gene showed that out of 105 initial calli bombarded, 45 calli were survived under hygromycin B 10 mg/L resulted in 42.8% putative transgenic calli frequency (Table 7) (Figure 35). While non-transgenic calli ultimately

died. It was concluded that calli and plants possessing the *Hpt* gene were resistant to the application of hygromycin B.

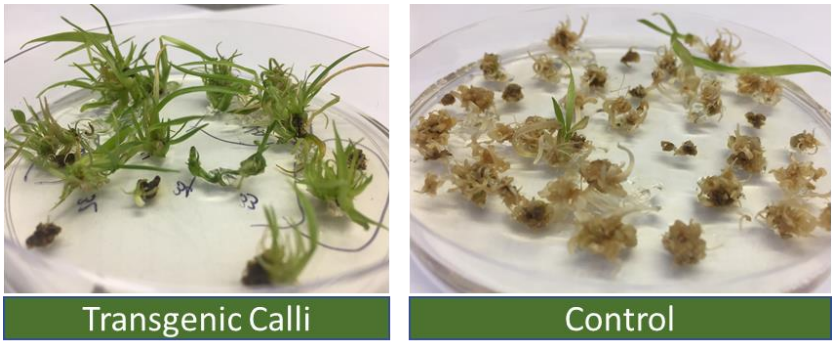

Figure 35. The regenerated calli after exposure of hygromycin B 10 mg/L; Transgenic calli conferring Hpt gene were survived, and the control calli died.

4.4.3.2 Confirmation of T0 Plants conferring the *Ds* construct

Both pSP-DsUbibar and pSPWDV-ActIDsUbibarDsGus constructs were transformed into the oat calli. The regenerated plants were molecularly analyzed using PCR with QNosF and QDS3R (Table 6) to amplify the 473 bp of Ds element in the 3' region. Of 60 calli transformed with pSP-DsUbibar, seven plants were regenerated, and no plants were positively detected containing the *DS* element as shown in the picture line 2-9 (Figure 36).

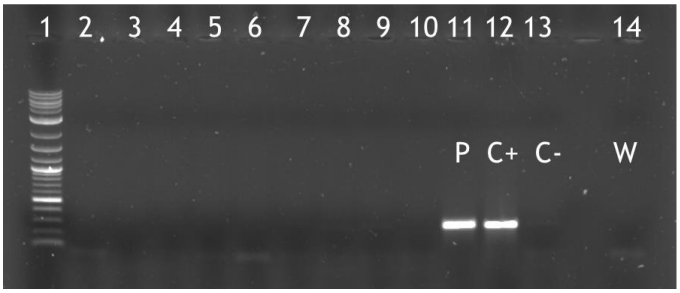

Figure 36. The PCR experiment of T0 plants transformed with pSP-DsUbibar; Line 1: ladder, Line 2-8: T0 Plants. P: plasmid, W: water, C+: positive control, C-: negative control.

Whereas, calli transformed with pSPWDV-ActIDsUbibarDsGus successfully regenerated three plants containing *Ds* element confirmed by the 490 bp band size amplification in 5’ and 473 bp band in 3' region. The primers used for the amplification in 5' and 3' area were DS5F and Ubi.Intr5R (Figure 37A) and QnosF and QDS3R (Figure 37B), respectively. The samples that considered transgenic plants were shown in electrophoresis picture lines 1, 9, and 10, named D6, Y1, and Y2. Even though samples numbers 2 and 3 demonstrated the amplification in 3' region, they did not amplify in the 5' Ds region; hence these samples are not considered the mutant plants.

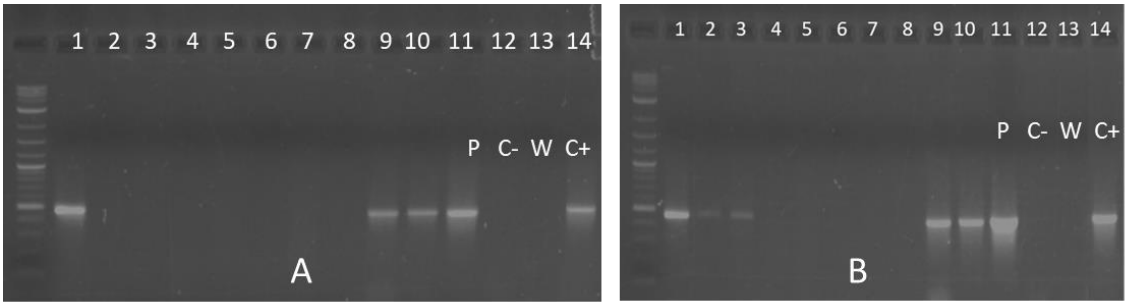

Figure 37. The pSPWDV-ActIDsUbibarDsGus construct was introduced into the genome. (A) Represented the amplification in the 5' of Ds construct while (B) showed 3' of Ds construct. Line numbers 1, 9, and 10 were the T0 mutant plants. P: plasmid, W: water, C+: positive control, C-: negative control

4.4.3.3 Confirmation of the transformation in T0 Plants conferring *Ac* construct

As the pCambiaUbiAcnos construct does not contain selection genes, pAct1HPT-4 vector containing *hygromycin resistance* (*hpt)* gene and pAHC20 vector harboring the *bar* gene were used as a donor. T0 plants co-bombarded with pAct1HPT-4 vector were tested molecularly to identify the insertion. PCR was done using primer HPTF and HPTR (Table 6) to amplify the region at 1000 bp. Out of 45 plants, 29 plants amplified at the desired band, as shown in Figure 38. Those were named H3.1, H3.2, H3.3, H3.4, H4.1, H4.3, H5.1, H5.3, H5.5, H5.7, H6.2, H9.1, H9.2, H10.3, H11.1, H11.2, H12, H13.1, H13.2, H13.3, H14, H16.1, H17.1, H18, H19.1, H19.3, H19.4, H19.5, H20.2.

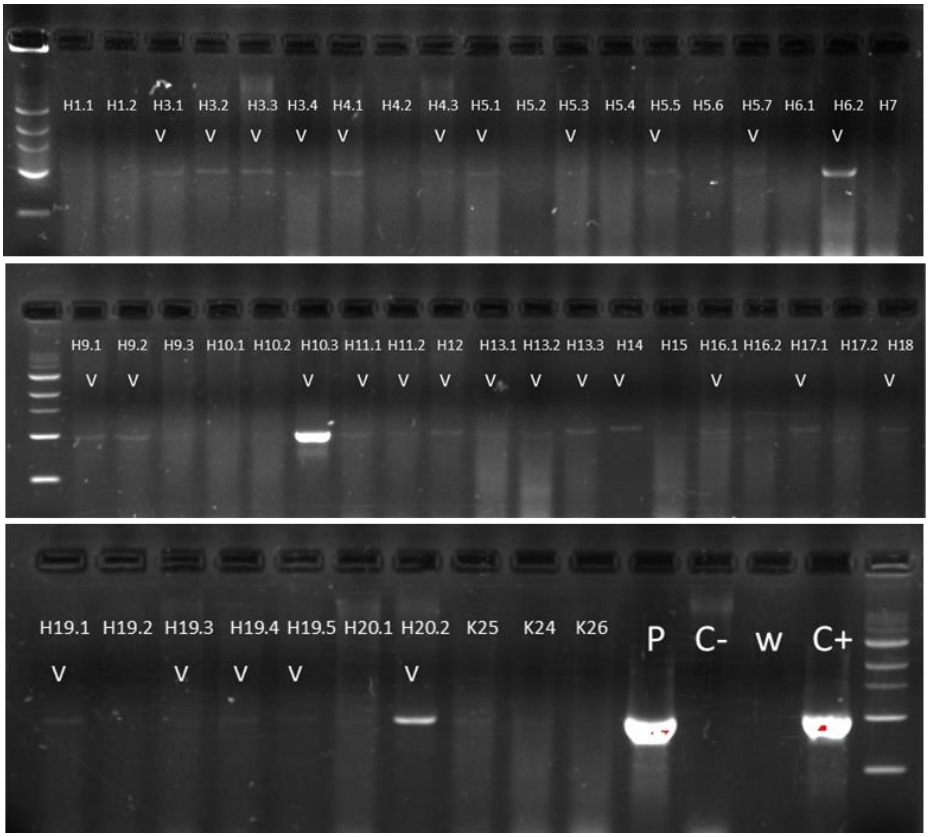

Figure 38. Molecular analysis of plants conferring Hpt genes. H3.1, H3.2, H3.3, H3.4, H4.1, H4.3, H5.1, H5.3, H5.5, H5.7, H6.2, H9.1, H9.2, H10.3, H11.1, H11.2, H12, H13.1, H13.2, H13.3, H14, H16.1, H17.1, H18, H19.1, H19.3, H19.4, H19.5, H20.2 were putative transgenic plants, P: plasmid, W: water, C+: positive control, C-: negative control.

To check the *Ac* insertion, samples were analyzed using primer AC3 and AC5, which amplified the transposase coding region. The amplification was demonstrated at 852 bp. Out of 45 plants, eight plants have *Ac* insertion (Figure 39), in which 6 of them also conferring the *hpt* genes; those were H3.2, H9.1, H10.3, H13.3, H12, and H17.2.

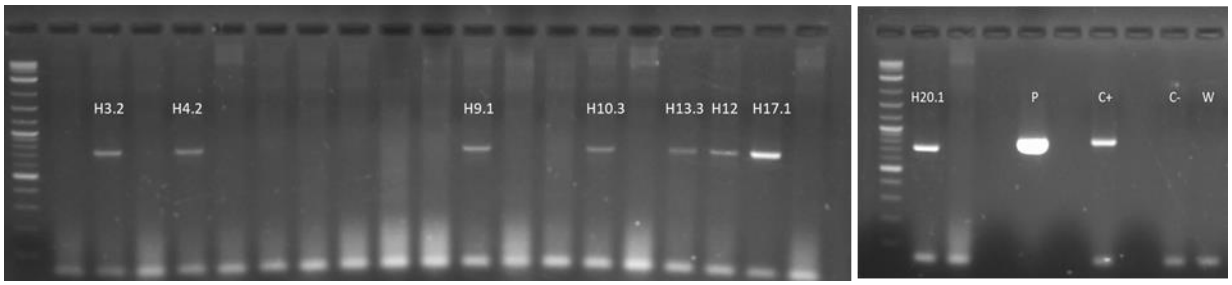

Figure 39. Molecular analysis of T0 plants conferring Ac elements co-bombarded with Hpt vector. H3.2, H9.1, H10.3, H13.3, H12, and H17.2 were putative Ac transgenic plants, P: plasmid, W: water, C+: positive control, C-: negative control.

Another *Ac* co-transformation was conducted with the pAHC20 construct, which conferred the *Bialaphos resistance* (*Bar*) gene. Five plants conferred the *Ac* insertion named as Q3, Q4, Q5, Q6, and Q9 (Figure 40). To resume, we have successfully created 11 total mutants possessing the *Ac* element.

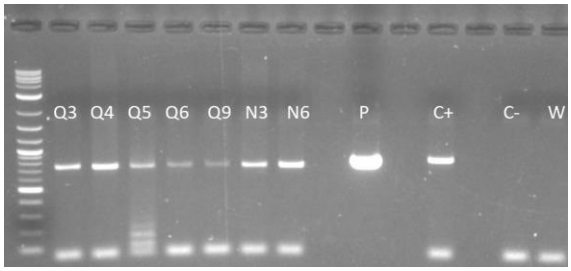

Figure 40. Molecular analysis of T0 plants conferring Ac elements co-bombarded with Bar vector. Q3, Q4, Q5, Q6, Q9, N3, N6 were Ac putative transgenic plants, P: plasmid, W: water, C+: positive control, C-: negative control.

4.4.3.4 Confirmation of T0 Plants conferring *Ac/Ds* activation tagging *Vec8* and *Ac* construct

In this part, the vector pWBVec8-DsUbiBarUbiDsGUS (Vec8) and pCambia-UbiAcnos were introduced. *Ac* fragment confirmation was performed using AC3, and AC5 primer resulted in 2 transgenic plants in the T0 stage, labeled as N3 and N6 Figure 40. Molecular analysis of T0 plants conferring Ac elements co-bombarded with Bar vector. Q3, Q4, Q5, Q6, Q9, N3, N6 were Ac putative transgenic plants, P: plasmid, W: water, C+: positive control, C-: negative control.

Figure 40).The vec8 construct's confirmation was observed using AC5 and Ubi.intr5R, which amplify the polyubiquitin region and 5' fragmented Ac region. The gene was amplified on 1 kb, as shown in Figure 41A, confirming the insertion of vec8. The *Gus* gene's expression in the Vec8 acts as the indicator of the transposition event.

4.4.3.5 Observation of Transposition Activity

The successful introduction of vec8 and Ac vectors (Figure 40 and Figure 41A) into the genome induces the transposition activity in the oat genome. Transpositions were confirmed with primer UbiDs-uidAF2, and UbiDs-uidAR2 (Table 6) occurred at 1 kb. The primer amplified the *Ds* region and vector backbone in the positive control and plasmid control. However, the amplification was unsuccessful as Ds moved away from the backbone, as observed in N3 and N6 transgenic lines (Figure 41B). This analysis confirmed the excision of *Ds* from the original location.

Transposition activity was also confirmed by histochemical analysis using Gus assay in seeds and young leaf plants, as shown in Figure 42. During the transposon activity, the Ubi promoter linked to the *Ds* element was excised out along with the *Ds* element that stops the *Gus* gene expression. Therefore, it provided a good indicator of transposition activity. Gus Assay was conducted, and observations were made after 6 hours of imbibition for seeds and three days

after for young leaf samples. Transposition activity was confirmed in both N3 and N6 plants, as no blue color appeared in the seeds and leaves (Figure 42B and C). Whereas, Positive control consisting only vec8 showed blue color expression, explaining no transposition activity occurred (Figure 42D).

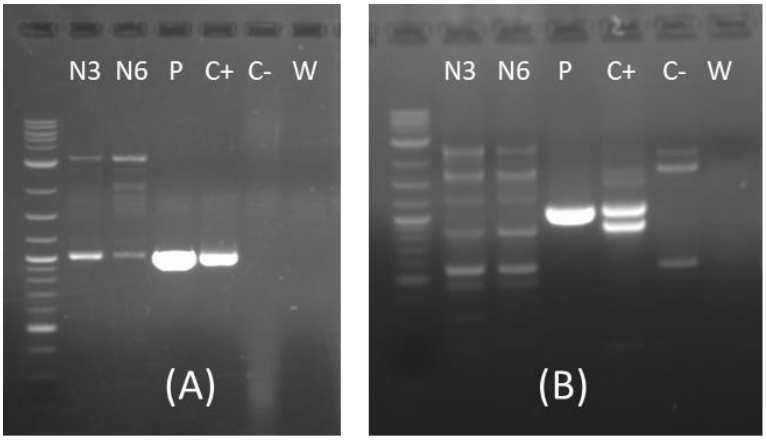

Figure 41. The molecular analysis of co-transformation event of UbiAcNos and Vec8 construct. Figure A: Vec8 confirmation and Figure B: transposition event. N3 and N6: Transgenic plants, P: plasmid, W: water, C-: negative control.

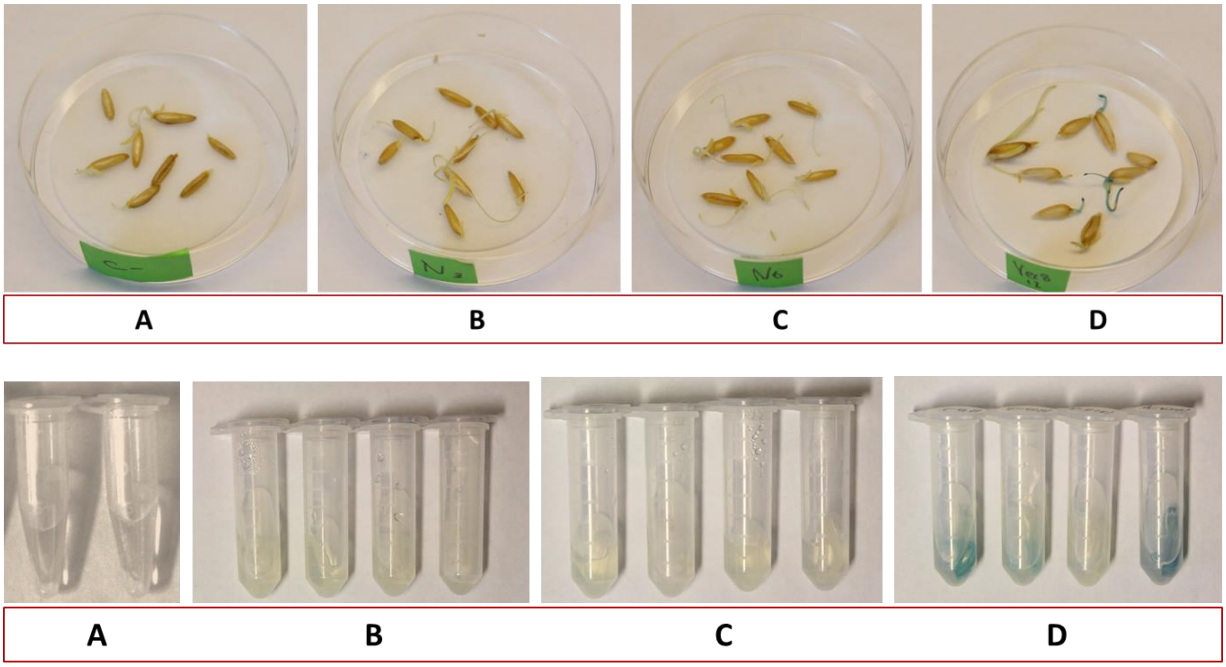

Figure 42. Biochemical gus assay analysis in seeds and leaf of plants harboring co-transformation event of UbiAcNos and Vec8 construct. A: non-transgenic plant, B. N3 transgenic plant, C: N6 transgenic plant, D. Positive control (plan 12-12).

Table 7. The record of transformation in oat plants using different constructs

Construct	Number of calli	Putative transgenic Calli Frequency (%)	Transgenic plants in T0	Plant Transformation Efficiency (%)	Selection
pSPWDV-ActIDsUbibarDsGus	62	37.1% (23 of 62)	3	13.04% (3 of 23)	Bialaphos 3 mg/L (2 rounds)

pSP-DsUbibar	60	11.6% (7 of 60)	0	0%	Bialaphos 3 mg/L (3 rounds)
pCambia-UbiAcnos + pAct1HPT-4	105	42.8% (45 of 105)	8	17.7% (8 0f 45)	Hygromycin 10 mg/L (1 round)
pAHC20 & pCambia-UbiAcnos	64	14.1% (9 of 64)	5	55. 5% (5 of 9)	Bialaphos 3 mg/L (3 rounds)
pWBVec8-DsUbiBarUbiDsGUS & pCambia-UbiAcnos	65	4.6% (3 of 65)	2	66.6 % (2 of 3)	Bialaphos 3 mg/L (3 rounds)

4.5 Discussion

Development of tissue culture and transformation system in diploid *A. strigosa* genome

The transposable elements mutagenesis approach has been utilized in many crops species such as rice, wheat, and barley to identify agronomically essential genes (Ayliffe *et al.*, 2007; Fridborg *et al.*, 1999; Jiang *et al.*, 2003; Jiang *et al.*, 2007; Koprek *et al.*, 2000; Takumi, 1996). It was reported that plants transformed with transposable elements conferred improved agricultural traits such as yield enhancement, tolerance to abiotic stresses, and plant resistance against pathogens (Jiang *et al.*, 2007). Many transposable elements have been characterized and identified in which maize *Ac/Ds* elements are one of those that have been described in detail and utilized in various plants (Carter *et al.*, 2013; Du *et al.*, 2011; Guiderdoni & Gantet, 2012; Jiang *et al.*, 2003; Koprek *et al.*, 2000; Lazarow & Lütticke, 2009; McClintock, 1951)

It was reported that the maize *Ac/Ds* tagging approach was successfully introduced in common oat (*Avena sativa*) (Mahmoud, 2019). However, no report was published about this work in *A. strigosa* diploid oat. Here, we are the first to report the introduction of the maize *Ac/Ds* functional genomics approach in *Avena strigosa*. We aimed to explore and identify novel genes, especially genes related to β-glucan synthesis and regulation. *A. strigosa* was chosen because of its significant amount of β-glucan (Welch *et al.*, 2000), availability of complete diploid As genome sequencing information (Maughan Peter *et al.*, 2019), and its benefits to overcome the gene redundancy issue on hexaploid oat (Comai, 2005).

An established and optimized tissue culture and transformation system are required for genetic transformation and functional genomic approaches. We successfully launched the

tissue culture and transformation system in diploid *A. strigosa*. Green calli produced from immature diploid oat embryo was good plant material for oat transformation due to a high transformation efficiency using this tissue. Successes in the green calli system have been reported from other plant species, including barley (Cho *et al.*, 1998) and common oat (Cho *et al.*, 1999).

There are several benefits of using regenerative calli for transformation system. They required only a small number of embryos to produce a large number of calli within a short time. After the calli are formed, they could be sub-cultured and maintained even after a long time producing enough sources for transformation experiments (Cardinal *et al.*, 2016). Furthermore, being kept sterilized, calli continuously provide clean and healthy starting material (Kulkarni *et al.*, 2007).

Investigation of transient and stable maize *Ac/Ds* transformation in diploid *A. strigosa*

With the development of an oat tissue culture system, it is now possible to develop transposable element mutagenesis approaches. Several *Ac/Ds* elements were introduced into *A. strigosa* genome using particle gen gun mediated transformation. Transient expression was first conducted to determine the maize *Ac/Ds* activity in *A. strigosa*; since no report was published yet. Calli were bombarded with *Ac* construct (pCambia-UbiAcnos) and *Gus*-trapped (pSPWDV-ActIDsUbibarDsGus) construct. The presence of *Ds* in between the Actin promoter and *Gus* gene blocked the expression of *β-glucuronidase*, an enzyme from *Escherichia coli* that gave specific color or fluorescence when it is incubated with a chromogenic (color-generating) substrate (Jefferson *et al.*, 1986). The *Ac* construct provided the transposase enzyme that induces the transposition activity by recognizing the Terminal Inverted Repeat (TIR) site (Lazarow & Lütticke, 2009). When pCambia-UbiAcnos and pSPWDV-ActIDsUbibarDsGus constructs were successfully integrated into the genome, the Ac transposase enzyme catalyzes the excision of *Ds* element, leading the Actin promoter to initiate the *β-glucuronidase (Gus)* gene expression (McElroy *et al.*, 1997). Gus activity can be histochemically detected when the calli expressing *gus* gene interacts with 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) solution, transforming blue color (Cardinal *et al.*, 2016). We confirmed that the maize Ac/Ds system was functional in *A. strigosa* genome through this transient assay.

With the successful transient assay, we subsequently investigated the stable *Ac/Ds* transformation in the *A. strigosa* genome using various *Ac/Ds* constructs with the previously described protocol for particle gene gun bombardment (Cardinal *et al.*, 2016; Mahmoud, 2019). Due to the optimized tissue culture and transformation system, regeneration frequency was highly attained up to 42.8% with up to 66.6% of transformation efficiency. It is reported that regeneration frequency was influenced by the number of calli that successfully take the transgene in the genome, combined with the number of cells that are totipotent and able to generate fertile plants (Cardinal *et al.*, 2016). The selection was conducted under herbicide Bialaphos (IGNITE, Bayer Crop, Canada) 3 mg/L , and Hygromycin B (Sigma) 10 mg/L. It showed no much difference in using either Bialaphos or Hygromycin, indicating that both are good selective agents in oat transformation.

Preliminary molecular screening analysis by PCR on regenerated plants demonstrated that three plants harbored *Ds* insertion, 11 plants carried *Ac* insertion, and two plants had double *Ac* and *Ds* insertion. It is suggested that the success of any in vitro mutagenesis approach depends on some factors such as a) reproducible tissue culture process, b) optimized transformation technique, and c) effective selection on the mutant's lines in the population (Jain, 2006; Mohan Jain, 2006).

Investigation of transposition activity in plants with *Ac/Ds* elements

The transposition activity was observed in two transgenic lines co-bombarded with pWBVec8-DsUbiBarUbiDsGUS and pCambia-UbiAcnos, as postulated that when *Ac* and *Ds* elements are together in the genome, the transposition activity could happen (Cardinal *et al.*, 2016). Ayliffe *et al.* (2007) also reported a similar observation who successfully established high level of *Ds* transposition from multiple launch sites in barley. It was suggested that the efficient *Ds* excision was dependent on the transposase enzyme level; thus, *Ac* elements were an essential donor for the transposition event (Sugimoto *et al.*, 1994). Some studies found that transposition event was correlatively in proportion with *Ac* donor. An elevated level of Ac transposase would lead to higher excision frequencies (Swinburne *et al.*, 1992). However, McElroy *et al.* (1997) discovered a converse result, that a less copy number of Ac transposase leads to higher transposition events. It seemed to be a species-dependent; for instance, the higher copy number that correlates with higher excision was found in Tobacco (Hehl & Baker, 1990) and Arabidopsis (Bancroft & Dean, 1993). Conversely, it was found in maize

(McClintock, 1951). Whereas, in our experiments, we discovered that less amount of *Ac* donor plasmid (0.5 µg) was sufficient to generate transposition activity *in A. strigosa*.

In summary, having transgenic plants with different *Ac* and *Ds* genomic resources is advantageous for researching different purposes. Ds mutants line lacking transposase enzyme, for example, could be reactivated by crossing with plants harboring transposase enzyme, simplifying a complex and lengthy transformation system (Koprek *et al.*, 2000). This system enables the experiments maintained for an extended period of time. In addition, many mutants events can be created within a short time, enabling to target and isolate many genes and avoid the somaclonal variation caused by the extensive tissue culture periods (An *et al.*, 2005; Jeong *et al.*, 2002). This could be an essential approach to characterize and identify the gene function, especially those related to health-promoting traits.

4.6 Conclusion

Tissue culture and transformation system has been standardized in diploid *A. strigosa*, providing essential information for subsequent functional genomic approaches. Green calli were reported as good starting material for transformation and could be maintained for an extended period. The transient assay has confirmed that maize *Ac/Ds* elements worked successfully in the oat genome, identified with histochemical gus assay experiment. Subsequently, a stable transformation of various *Ac/Ds* constructs has been generated using particle gen gun bombardment. It is reported as the first effort ever to genetically transformed *A. strigosa* diploid oat with *Ac/Ds* transposable elements. Due to the well-established tissue culture and transformation system, a high putative transgenic calli frequency (up to 42.8%) and transformation efficiency (up to 66.6%) was achieved. Molecular characterization confirmed that three plants harbored *Ds* insertion, 11 plants carried *Ac* insertion, and two plants had double *Ac* and *Ds* insertion. Utilizing histochemical gus assay, the transposition activity was also observed in plants conferring both *Ac* and *Ds* elements.

To conclude, generating mutant lines using the transposable elements approach is a promising mutagenesis strategy, especially in crops where the tissue culture and transformation system is laborious and time-consuming. Transposon-mediated genomic resources are valuable resources for exploring genes' function in the oat genome, particularly to reveal other genes related to important agronomical traits.

CHAPTER V: General Conclusion, Future directions, and Contribution to science

General conclusion

Our study developed functional genomic approaches to explore and identify genes associated with β-glucan in oat. Using the barley *TLP8* gene, we successfully retrieved oat *TLP8* ortholog. The RNAi construct was successfully generated and introduced in hexaploid *A. sativa* green calli using particle gene gun bombardment. Transformants were selected with herbicide bialaphos (3mg/L) and glufosinate liberty (0.2%). It yielded a high putative calli frequency (49.8%) with 7.4% transformation efficiency. Molecular analysis confirmed that 15 plants in the T0 generation and two plants in the T1 generation conferring RNAi transgene. Expression analysis conducted in T2 RNAi transgenic seeds demonstrated a significant reduction of *TLP8* transcript. However, a converse expression was seen during germination, which needs further investigation.

In addition, the maize *Ac* and *Ds* transposable elements were successfully introduced into the high β-glucan diploid oat (*A. Strigosa*) to create a transposon-mediated for exploration of other genes associated with β-glucan content. The tissue culture and transformation system in this species has been developed and standardized. The transient assay has confirmed that maize *Ac/Ds* elements were functioning in the *A. strigosa* oat genome, identified with histochemical gus assay experiment. Subsequently, a stable transformation of various *Ac/Ds* constructs has been generated using particle gen gun bombardment. High putative calli frequency (up to 42.8%) with 66.6% of transformation frequency was observed in *A. strigosa*. Molecular characterization confirmed that three plants harbored *Ds* insertion, 11 plants carried *Ac* insertion, and two plants had double *Ac* and *Ds* insertion.

To conclude, our experiment has successfully generated TLP8-knockdown transformants and *Ac/Ds* transposon transformants, which are valuable for future exploration and identification of genes, particularly genes associated with β-glucan in oat.

Future Direction

To better understand genes related to β-glucan synthesis and regulation, the following studies are required in the future:

1. Mixed-linked β-glucan assay to calculate the β-glucan content in oat. It will help to understand the association of TLP8 and β-glucan content.
2. Generating a homozygous line to ensure the stable integration and inheritance of RNAi transgene, especially in transgenic line RP 17.1-3, since homozygous line RP 84-18 has been generated.
3. Investigation of germination rate in transgenic homozygous lines to validate the function of *TLP8* during germination.
4. Molecular analysis of T1 progeny of *Ac/Ds* transformants to confirm *Ac/Ds* elements' heritability.
5. Identification and characterization of transposon-tagged genes using various PCR methods such as iPCR, TAIL-PCR, or HeTAIL-PCR. The information of these genes is valuable for gene expression study, especially genes for agronomical quality improvement.
6. Generating a library of stable *Ac* and *Ds*-tagged transformants for further use in the breeding program.

Contribution to Science

My study could contribute to science by providing essential resources for functional genomic studies as below:

1. Knocked-down TLP8 lines of hexaploid *Avena sativa* could lead to a better understanding of the TLP8 association with β-glucan synthesis and regulation in oat.

2. Genetic transformation and *Ac/Ds* based-transposon system developed in *Avena strigosa* will assist in exploring and identifying genes, particularly genes associated with β-glucan content.

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