Mycorrhizal colonization in wheat: phenotypic and genotypic analysis

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Abstract

Wheat (*Triticum spp.*) is the third most produced cereal crop, being planted on more than one sixth of global cultivated farmlands, and providing 40% of world grain production. Numerous biotic and abiotic challenges can limit its production. Mycorrhizal-colonization of roots is a promising approach for mitigating stresses, has been studied in different plants (ex. rice, pepper, citrus, etc.) and has proven to be beneficial in aspects such as: increasing water absorption, enhancing mineral uptake and improving abiotic stress tolerance. Studies on this mutualistic relationship have been limited in wheat and information regarding alternation of antioxidant capacity of various wheat grain under mycorrhizal colonization is scarce.

This project investigated the influence of four mycorrhizal strains colonization on four different spring wheat genotypes under three salt treatments. In the first study, changes in root morphology and plant biomass were measured and analyzed to identify the best performing mycorrhiza strains and wheat genotypes. In the second study, grains of those mycorrhizal-colonized wheat varieties were collected to investigate 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity and total phenolic content (TPC). The third study identified stable transposon mutants in wheat, which could be further used to study mycorrhizal wheat interaction related genes.

Results of the first greenhouse experiment demonstrated that wheat inoculated by arbuscular mycorrhizal strains *Funneliformis mosseae* and *Rhizophagus irregularis* mitigated yield losses caused by increased salinity stresses and strengthened root systems in comparison to non-inoculated plants. The wheat variety FL62R1 had the best performance in most variables tested. Salinity stress had significant negative effect mainly on root surface area and root dry weight in all wheat-mycorrhizal combinations. Results from a modified greenhouse trial of this stress

experiment were consistent with the first study, showing *R. irregularis* colonization in plant roots seemed to help wheat develop stronger root systems. Enhanced root length, root total volume, fresh and dry root weight and root to shoot ratio were all demonstrated in inoculated plants relative to controls.

Analysis results of grain antioxidant capacity clarified that 13NQW1265, the purple wheat strain, possessed the strongest antioxidant activity among the four selected wheat genotypes, representing as getting the highest value in the DPPH assay and containing maximum TPC in grains. A comparison between grain TPC in mycorrhizal inoculated and non-inoculated wheat (control) demonstrated that inoculation of the commercial strain *Myke* (*Rhizophagus irregularis*) negatively decreased grain TPC in FL62R1 wheat but TPC were significantly improved in wheat 13NQW1265 with its roots colonized by *Funneliformis mosseae*. Moreover, a significantly positive correlation was uncovered between these two indexes of antioxidant capacity, DPPH scavenging capacity and TPC.

In the third study, 32 out of 48 *Ac/Ds* T_1 transgenic wheat plants were identified to contain both *Ac* and *Ds* constructs and *Ds* transposition occurred in four putative plants. These 4 lines would be valuable in further research to understand wheat-mycorrhizal interaction.

Résumé

Le blé arrive au troisième rang des cultures céréalières dans le monde, couvre le sixième des terres cultivées et constitue 40% de la production de grains et ce même si de nombreux embûches biotiques et abiotiques limitent sa production. L'impact des symbioses mycorhiziennes sur la tolérance aux stress a été étudié sur plusieurs plantes (ex. riz, poivron, agrumes etc.) et les bénéfices associés à de meilleurs régimes hydriques, une absorption accrue de minéraux et une tolérance aux stress abiotiques. Ce mutualisme a été étudiée chez le blé toutefois peu de travaux traitent de l'impact des mycorhizes sur la composition en antioxydants des grains de blé.

Ce projet traite de l'impact de quatre souches mycorhiziennes sur quatre génotypes de blé soumis à trois traitements de salinité. L'analyse de la morphologie racinaire et de la biomasse ont d'abord permis de sélectionner la souche fongique et le génotype les plus performants; la mesure des phénols totaux (TPC, DPPH (2,2-diphenyl-1-picrylhydrazyl) a ensuite permis d'évaluer la capacité des grains à piéger les radicaux libres. L'identification d'un transposon mutagène du blé susceptible d'éclairer sur les relations mycorhiziennes inter géniques chez le blé a été entreprise.

L'inoculation mycorhizienne du blé avec les souches *Funneliformis mosseae* et *Rhizoglomus irregulare* atténuent l'effet nocif de la salinité sur la biomasse des plantes colonisées et renforce leur système racinaire. La variété de blé FL62R1 présente les meilleures performances. Le stress salin affecte négativement la surface totale des racines et leur biomasse sèche. La répétition des essais a confirmé ces résultats et indiqué un impact significatif de *R. irregulare* sur la longueur et le volume racinaire, biomasse fraîche et sèche des racines et le rapport racine et tige.

Le génotype de blé pourpre 13NQW1265 a révélé une activité antioxydant supérieure à celle des trois autres génotypes avec le meilleur taux de DPPH et de plusieurs TPC dans les grains. La comparaison entre blés mycorhizés et témoins non mycorhizés révèle un effet négatif de

l'inoculant commercial *Myke* (*R. irregulare*) sur le taux de TPC chez le génotype FL62R1 mais à l'inverse, une augmentation du taux de TPC lorsqu'inoculé avec *F. mosseae*. De plus, une corrélation positive significative existe entre la capacité de piégeage et les TPC.

Enfin, 32 des 48 *Ac/Ds* T_1 plantes transgéniques étudiées contiennent les éléments *Ac* et *Ds* et la transposition de l'élément *Ds* advient chez quatre d'entre elles. Ces quatre lignées pourront servir ultérieurement à la compréhension des interactions blé-mycorhizes.

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Contributions of authors

Chapter 3: the response of spring wheat cultivars to arbuscular mycorrhizal (AM) colonization under salinity stresses

This manuscript was co-authored by the candidate and Prof. Jaswinder Singh and Dr. Timothy Schwinghamer in Department of Plant Science, McGill University and Prof. Shahrokh Khanizadeh and Prof. Yolande Daplé from Eastern Cereal and Oilseed Research Centre of Agriculture and Agri-Food Canada. The primary author of manuscripts, Daishu Yi, performed the experiments, data collection and analysis. The research question and study design was provided by Prof. Shahrokh Khanizadeh, Prof. Jaswinder Singh and Prof. Yolande Daplé. Dr. Timothy Schwinghamer provided help in data analysis.

Chapter 4: Response of spring wheat (*Triticum aestivum* L.) varieties to arbuscular mycorrhizal colonization

This second manuscript was co-authored by the candidate, Prof. Jaswinder Singh and Dr. Timothy Schwinghamer in Department of Plant Science, McGill University, together with Prof. Shahrokh Khanizadeh and Prof. Yolande Daplé from Eastern Cereal and Oilseed Research Centre of Agriculture and Agri-Food Canada. Daishu Yi, the primary author of manuscripts, performed the experiments, data collection and analysis. The research idea was provided by Prof. Shahrokh Khanizadeh, Prof. Jaswinder Singh and Prof. Yolande Daplé. Dr. Timothy Schwinghamer provided help in data analysis.

Chapter 5: Comparison of total phenolic content and antioxidant capacity of mycorrhizal-colonized white, red and purple spring wheat (*Triticum aestivum* L.) genotypes

This third manuscript was co-authored by the candidate and Prof. Jaswinder Singh and Dr. Timothy Schwinghamer from Department of Plant Science, McGill University, together with Prof. Shahrokh Khanizadeh, Prof. Yolande Daplé and Dr. Xuelian Wang in Eastern Cereal and Oilseed Research Centre of Agriculture and Agri-Food Canada and Prof. El-Sayed Abdel-Aal from Guelph Research and Development Centre, Agriculture and Agri-Food Canada. Daishu Yi, the first author of manuscripts, performed the experiments and data collection and analysis. The research idea was provided by Prof. Shahrokh Khanizadeh, Prof. Jaswinder Singh and Prof. Yolande Daplé. Prof. El-Sayed Abdel-Aal provided help on TPC and DPPH scavenging capacity extraction and measurement. Dr. Timothy Schwinghamer provided help in data analysis.

Chapter 6: Identification of stable *Ds* transposon mutants in wheat

The final manuscript was co-authored by the candidate and Prof. Jaswinder Singh, Department of Plant Science, McGill University. Daishu Yi, the primary author of manuscripts, performed the experiments and data analysis. The research idea was provided by Prof. Jaswinder Singh.

List of Abbreviates

Ac/*Ds*	Activator-Dissociation system
AFLP	Amplified Fragment Length Polymorphism
AM	Arbuscular Mycorrhiza
AMF	Arbuscular Mycorrhizal Fungi
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
CS42	Chinese Spring line 42
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
EcM	Ectomycorrhizas
EU	Europe Union
Gb	Gigabase pair
GM	Genetic Modification
GMO	Genetically Modified Organism
LINEs	Long Interspersed Nuclear Elements
LTR	Long Terminal Repeat
MAS	Marker Assisted Selection
Mb	Mega base pair
MGE	Mobile Genetic Element
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RCBD	Randomized Complete Block Design
ROS	Reactive Oxygen Species

RPLP	Restriction Fragment Length Polymorphisms
SAGE	Serial Analysis of Gene Expression
SCLB	Southern Corn Leaf Bright
SDS	Sodium Dodecyl Sulphate
SINEs	Short Interspersed Nuclear Elements
SNP	Single Nucleotide Polymorphisms
SSR	Simple Sequence Repeats
TE	Transposable Element
TER	Tris Acetate RNase
TPC	Total Phenolic Content
VAM	Vesicular Arbuscular Mycorrhizas
WGS	Whole Genome Sequencing

Chapter 1

General Introduction

1.1 Introduction

Wheat (*Triticum spp.*), known as one of the first domesticated crops, plays a pivotal role in human civilization. With a global production of over 0.71 billion tons, wheat ranks as the third most produced crop after maize and rice and feeds approximately half of the global population (FAOSTAT 2013; Gupta et al 2008). In addition, its importance in the caloric element of diets, wheat is also considered a donor of natural antioxidants and the consumption of wheat products prevents numerous chronic disease developments (Jacobs et al., 1995; Jacobs et al., 1998; Kasum et al., 2002; Meyer et al., 2000; Nicodemus et al., 2001; Tompson 1994). However, wheat production is now under severe challenges from abiotic and biotic stresses and therefore exploration of ways to alleviate stress-induced detrimental effects is of great importance. Many approaches have been discovered involving generation of tolerance varieties, improvement of soil conditions via application of various external compounds, etc. One promising path is the utilization of arbuscular mycorrhizal strains, using a natural, mutualistic symbiosis and studies into this wheat-mycorrhizal interaction are also of great value for their intrinsic addition to knowledge of this relationship. Transposons are also desirable tools for gene function identification and additional knowledge of their role in wheat quality influences is also important.

The long term objective of this research project is to investigate the relationship between mycorrhiza and wheat genotypes under salinity stress from both phenotypic and genotypic aspects. In the first study, an evaluation was made on the responses of wheat verities to arbuscular mycorrhizal colonization under salt stress from both plant biomass and root morphology aspects.

The initial study was supported by a second, modified experiment on wheat-mycorrhizal interaction under normal conditions. In the second study, alternation of antioxidant capacity in wheat when inoculated with diverse mycorrhizal strains was analyzed and compared. A third study focused on the screening of transposon-based mutants and putative transposition lines, and could lead to subsequent functional genomic research. It could be considered the initial step for wheat-mycorrhizal interaction understanding from a molecular perspective, and used for additional, novel gene identification and understanding. The research presented below will illuminate a deeper understanding of wheat-mycorrhizal interaction.

1.2 Hypotheses

The research project was designed and conducted based on the following hypotheses:

- Arbuscular mycorrhizal association enhances salt tolerance in wheat species
- Tolerance against oxidative stress is improved in mycorrhizal wheat
- Transposon mutants can be of great use in wheat gene identification

1.3 Objectives

Three main objectives were set based on those hypotheses:

- Evaluation of biomasses and root morphology of different wheat varieties with different mycorrhiza strains under salinity stress
- Detection and comparison of total phenolic content (TPC) and DPPH scavenging capacity in different wheat genotypes inoculated by different mycorrhizal strains
- Identification of stable *Ds* transposon mutants in wheat for the understanding of the interaction between arbuscular mycorrhizal fungi and wheat

Chapter 2

Literature Review

2.1 Historical and economic importance of wheat

The importance of wheat (*Triticum spp.*) cannot be disputed. Nowadays, wheat has edged itself to the top three most produced cereal crops together with maize and rice, which is mainly attributed to its high adaptability, unrivalled range of cultivation and considerable high yield production (FAOSTAT 2013; Feldman, 1976; Shewry 2009). Planted on over 17% of global farming land, wheat successfully feeds nearly half of population around the world (Gupta et al 2008). In 2013, wheat had a global production of more than 0.71 billion tonnes, compared with 0.74 billion tonnes of rice and over 1 billion tonnes of maize (FAOSTAT 2013). In addition, it is of great importance in Canadian Agriculture. With a nationwide production of over 37 million tonnes and a net production value of more than 5 billion dollars, wheat ranks as the most produced crop in Canada, significantly overwhelming maize and barley's contributions (FAOSTAT 2013).

Known as one of the first agricultural-applied cereal crops, wheat has been domesticated and embedded into humanity since the beginning of "Neolithic Revolution", and the appearance of agriculture almost 10,000 years ago (Gupta et al., 2008; Lev-Yadun et al., 2000; Peleg et al., 2011). Sufficient evidences from botanical, genetic and archeological aspects have established that wheat (*Triticum spp*.) originated from the Fertile Crescent before spreading out globally from modern day Turkey and southern Syria, which is the same with other Neolithic founder plants including barley (*Hordeum vulgare* L.), bitter vetch (*Vicia ervilia L. Willd*.), pea (*Pisum sativum L*.) and chickpea (*Cicer arietinum L*.), etc. (Brown et al 2009; Feldman & Kislev 2007; Lev-Yadun et al 2000; Peleg et al 2011). Nowadays, as a consequence of the selection process, both natural and artificial, and other evolutionary forces, wheat has become globally distributed, ranging from 67°N

to 45°S. It is intensively planted in temperate and subtropical regions such as the Mediterranean and Western Asia, with a total acreage of over 216 million hectares (Brown 2010; FAOSTAT 2013; Gupta et al., 2008; Peng et al 2011). The top three wheat producing countries are China (122 M tons), India (94 M tons) and the United States (58 M tons); Canada ranks seventh with a total production of 27 million tons (FAOSTAT 2013).

Wheat plays an irreplaceable role in our daily life. Gluten proteins make wheat the crucial raw material for pasta, biscuits, bread and other baked products manufacture (Lamacchia et al 2014; Rooke et al 1999). Consumption of wheat products benefits human health by providing diverse nutrient including easily digested protein, beneficial lipids, abundant minerals, multiple vitamins as well as natural antioxidant components including phenolic acids (Fan et al 2008; Henderson et al 2003; Liu 2007). As reported in a survey conducted in Great Britain, daily intake of bread provides 15% of Fe, 11% of Zn and 17% of vitamin E (Fan et al 2008; Henderson et al 2003). In addition, wheat has medical value as well. It has been widely reported in previous studies that wheat consumption, especially whole grain wheat products, helps alleviate the incidence of detrimental diseases involving tissue cancers (Kasum et al 2002; Jacobs et al 1995), cardiovascular disease (Jacobs et al 1998; Thompson 1994), and diabetes (Mayer et al 2000), which is partially attributable to the abundant antioxidant components in wheat whole grain (Fergunson and Harris 1999). Furthermore, wheat by-products are valuable as they can not only be made into forage for livestock feeding, but also be turned into bio-energy/biofuels, to substitute fossil energy (Boros et al 2004; Rosenfelder et al 2013; Rosegrant 2008). Taking the case in the Europe Union (EU) as an example; in 2009, one third of the EU wheat production came to human dining tables, while the remaining wheat by-products generated during processing were consumed as livestock feed (FAOTAT 2012; Rosenfelder et al 2013).

2.2 The wheat genome and its origin

Wheat (*Triricum.* spp) is an enormous genus with three different polyploidy subgroups. A small portion of wheat species is diploid, such as Einkorn wheat (*T. monococcum*), possessing two sets of chromosomes (total number 14, 2n=14). Most wheat species, however, are complicated but stable polyploidies with either four sets of chromosomes (total number 28), representing by emmer wheat (*T. dicoccon*) and durum wheat (*T. durum*) or six sets of chromosomes (total number 42), composed of mainly spelt (*T. spelta*) and bread wheat (*T. aestivum*) (Lupton, 2014; Rivera and Obón, 2004). Among all domesticated wheat variations, the bread (*T. aestivum*) and durum wheats (*T. durum*) are the most important species commercially; accounting for almost 95% and 5% of the global wheat production respectively (Mayer et al., 2014; Peleg et al., 2011).

The bread wheat or the common wheat (*T. aestivum*) is the main species in the hexaploidy family. There are three sub-genomes A, B and D in bread wheat, making up seven homologous groups (Gupta et al., 2008; Mayer et al., 2014). Each homologous group contains three genetically related chromosomes from the A, B, and D sub-genomes, respectively (Gupta et al., 2008). As the consequence of highly conserved syntenic phenomenon and the existence of conserved sequences between homologous chromosomes in bread wheat, scientists widely believed that every gene identified should have two extra homologous copies in another two genomes, except for cases of gene deletion (Brenchley et al., 2012; Lagudah et al., 2001). The bread wheat contains a huge and intricate genome as a consequence of large number of co-existing genomes caused by polyploidization during domestication and the abundance of repetitive sequences (~90% of the full wheat genome is repeated, as compared to 22% in rice and 80% in maize) (Gill et al., 2004; Ma et al., 2004; Whitelaw et al., 2003). Bread wheat has a 16-17 Gb (AABBDD genome, in 42 chromosomes), amounting to 8 time of maize genome (~2500Mb), 40 fold that of rice (~430Mb)

and 120 fold the *Arabidopsis* genome (~130-140Mb) (Arumuganathan and Earle, 1991). Many researches have been done on the progenitors of A, B and D sub genomes and the evolution progress of the allohexaploid. Although there are still disputes about the ancestor of B genome, extensive acceptations have been acquired on the possible origin of wheat's full genome and evolution of bread wheat (Brenchley et al., 2012; Gill et al., 2004; Hetrick et al., 1993; Rief et al., 2005). To be specific, a natural hybridization and spontaneous chromosome doubling between the diploid *Triticum urartu*, the progenitor of the AA genome and an unknown diploid, the progenitor of the BB genomes contributed to the emergence of the tetraploid emmer wheat (*Triticum dicoccoides*) with a genome of AABB (Blake et al., 1999; Hetrick et al., 1993). A further polyploidization between the domesticated tetraploid emmer wheat and a diploid goat grass (*Aegilops tauschii*, DD genome) finally produced the hexaploid bread wheat varieties (Hetrick et al., 1993; Kihara, 1944; McFadden and Sears, 1946).

The Chinese Spring lines 42 (CS42) is the first bread wheat cultivar whose complete genome set has been decoded and published (Brenchley et al., 2012). To date, more than 94,000 genes have undergone in-depth study and been identified through orthologous gene comparison with other crops (Akhunov et al., 2012; Brenchley et al., 2012). The successful sequencing of the bread wheat genome enlightens our systematic understanding regarding cereal genomes in searches for more worthy genes and traits in breeding. However, there are still enigmas on the wheat whole genome, which worth further study and refined identification, especially when considering the intricate genome components and the genus' scientific and economic significance (Mayer et al. 2014).

2.3 Wheat breeding

Plant breeding is a subject that combines art and science, aiming at the creation and selection of plants with desirable traits (Poehlman and Sleper, 1995). Broadly, it is considered as changes

occured in plants as a consequence of human interference, either intentional such as crossing and more advanced molecular breeding or less intentionally, as seen in the advent of agriculture (Breseghello and Coelho, 2013). The primary function and goal of plant breeding is to explore and to select individual with pre-potent agronomic characteristics such as outstanding yield production, flavor and nutrient quality, tolerance against abiotic and biotic stresses, and/or ease of agronomic management (Breseghello and Coelho, 2013). Interestingly, instead of sticking to high education and professional requirement, breeding is a more flexible and multicultural practice that can be realized by both less professional farmers and well-educated plant breeders. Dating back 9,000 to 11,000 years ago, plant breeding entered the public consciousness simultaneously with the domestication of the first agricultural plants and could be considered as co-evolutionary products between plant domestication and human civilization (Breseghello and Coelho, 2013; Piperno et al., 2009). By artificially screening plants that meet special demands, the balance in natural population was broken and evolution was speeded up. In return, the modified plant population brought changes to human society, from both the aspects of population quantity and life modes (Breseghello and Coelho, 2013; Harlt and Clart, 1997). For example, the discovery of high-yield cultivars met food demands for more people and freed a portion of labor to focus on artistic creation and scientific research, which accelerated the appearance of modern life; the increased numbers of early-scientists further promoted breeding development and crop quality (Breseghello and Coelho, 2013). There is now a consensus that advanced breeding techniques speeds up agricultural development and that improved agriculture contributes to the multicultural development of human civilization (Harlt and Clark, 1997).

Generally, there are two dominant branches in the evolving plant breeding system: 1) the inchoate traditional or conventional breeding approaches, characterised by phenotypic selection through the

observed selection within natural variance or the generation of hybrid by intentional crossing between ascendant parent lines; 2) molecular breeding that enables directional selection through DNA and gene screening with the help of multiple molecular markers (Breseghello and Coelho, 2013). However, with the exponential development of the human population and increasing material and spiritual demands, modification of single trait is far from enough. A more complicated task aiming at improving multi-fold beneficial traits simultaneously has emerged, a task made extremely challenging by gene correlation phenomenon resulting from gene pleiotropy, physical linkage or population genetic structure (Falconer and Mackay, 1995; Harlt and Clark, 1997).

2.3.1 Traditional or conventional breeding

The traditional or conventional breeding can be broadly separated into two typical phases: the inchoate observation based selection within naturally existing varieties representing the origin of crops, the landrace and further developed cultivars (Breseghello and Coelho, 2013； Robert, 1929). Breeding uses intentional mating to dramatically increase allelic recombination rates to create infinite new valuable genotypes, to some extent, in almost all plant breeding programs (Breseghello and Coelho, 2013; Rasmusson and Philipps, 1997; Xu, 2010).

Plant breeding is initialized from the selection of naturally occurring mutants in the wild (Breseghello and Coelho, 2013). As the consequence of strong natural selection and human preference and demand selective pressures, a large amount of wild mutants dropped out from gene pools, facilitating the generation of early cultivars but undesirably declining gene diversity. This also resulted in loss of a vast of valuable wild types, especially wild types with now treasured resistance genes (Harlt and Clark, 1997; Tanksley and McCouch, 1997; Zamir, 2001). It has been widely proven that gene diversity was significantly decreased as a result of plant evolution and conventional breeding in crops such as rice, wheat and barley (Chesser, 1991). Landraces are plant

populations grown in certain regions. Being cultivated in constant region for years, landraces have been adapted to regional selective pressure and gain stable characteristics that help them fight against environmental stresses while meeting specialized demands from local people (Breseghello and Coelho, 2013). The interaction between stabilizing selection and directional selection finally contributes to the generation and evolution of a landrace. Stabilizing selection plays a role of keeping the identity of a landrace while the directional selection helps landraces adapt to environmental changes in their habitat slowly (Breseghello and Coelho, 2013). Considering the high gene diversity and adaptation to territorial environment, landraces are the most valuable gene resources available for further sustainable breeding and policies such as "ex-suit" and "in-suit"; used for the conservation of gene resources (Breseghello and Coelho, 2013). Further selection within landraces based on the discovery of law of inheritance emphasized the importance of pure line selection and gave rise to attention on homogeneity, valuable theories in plant breeding until now (Breseghello and Coelho, 2013). However, considering the existence of monomorphic alleles in most loci, pure lines are thought to be an less stable breeding resource, losing their advantages in face of biotic and abiotic stresses.

Even though abundant naturally occurring variances were selected and actively protected in inchoate breeding processes, the genetic heritage of domesticated varieties is no longer sufficient for further development. To explore more novel genetic resources, controlled mating arose to support increasing food and breeding demands. By intentional crossing, allelic recombination rates were dramatically increased, creating a myriad amount of new genotypes (Rasmusson and Phillips, 1997). Several crossing approaches have been discovered to take full advantages of these infinite gene resources, including pedigree breeding, ideotype breeding, population breeding as well as hybrid breeding (Breseghello and Coelho, 2013; Jensen 1988; Miah et al., 2013; Welsh 1981). For

example, pedigree breeding, the most familiarity dependent breeding method, has been a useful tool for the development of self-pollinating cultivars such as rice and barley, especially when qualitative trait such as disease resistance was considered (Breseghello and Coelho, 2013; Harlan et al., 1940; Miah et al., 2013). When quantitative traits such as yields were taken into consideration, ideotype breeding is a better choice under the hypothesis of gene correlation that target complex traits could be simply changed by modifying positively related traits (Donald, 1968; Peng et al., 2008; Rasmusson, 1991). One fatal limitation for this attractive breeding strategy is the undesirable correlation between traits, in which pre-breeding is required to break these genetic correlations (Breseghello and Coelho, 2013; Yuan et al., 2011). Population breeding or recurrent breeding is another traditional breeding method that aims to increase the frequency of desirable alleles. This breeding approach is widely used in cross-pollinated species like maize but also sometimes used in self-pollinated plants, represented by rice and wheat, etc. (Fujimaki, 1979; Jenkins et al., 1954). When applying a relatively larger population, it is required to offset the depletion of genetic diversity caused by repeated crossing events (Falk, 2010; Souza et al., 2000). Finally, hybrid breeding strongly relies on the theory of heterosis in hybrid offspring as a consequence of the existence of heterozygous loci (Breseghello and Coelho, 2013). Generally, the more divergent the parent lines are, the stronger heterosis the hybrid will have (Jeff et al., 2005; Moll et al., 1965). Thus, there are two main challenges in hybrid breeding that need to be overcame, the establishment of highly divergent parent lines and the reduction of heterosis decay in following generations (Schnable and Springer, 2013).

Generally, conventional breeding were powerful tools in primitive breeding process and are still of value in modern breeding systems, contributing a large amount of germplasm resources. However, there are fatal inevitable drawbacks and deficiencies in the traditional breeding systems.

First of all, cultivars selected through conventional breeding are more susceptible to biotic and abiotic stresses caused by salinity, drought, bacterial or fungi pests and pathogens, which result from the loss of curtain resistance genes when pursuing too much on high-yield and uniform crop management traits (Reif et al., 2005; Zamir, 2001). One typical example is the hasty explosion of southern leaf corn blight (SLCB) disease in United States in 1970s, causing disastrous production and economic loss, estimated to be over one billion US dollars, within one year (Agrios, 2005). Another critical shortcoming of the traditional breeding approach is considerable time requirement. Generally, it takes 5-15 years before a new cultivar could be released to public (Breseghello and Coelho, 2013; Miah et al., 2013). In this burgeoning world with exploding food demands from population stress and rapid increasing challenges from nature, a breeding process of 5-15 years is obviously an unaffordable expense. Therefore, the molecular breeding strategies offer a solution to more efficiently cater for to the demands of contemporary society.

2.3.2 Molecular breeding

Molecular breeding is an advanced breeding system compared to the primitive, time-consuming conventional breeding techniques. It emerged with the development of molecular biology tools (Moose and Mumm, 2008; Voosen, 2009) and the primary difference between conventional breeding and modern molecular breeding lies in the fact that molecular breeding focuses on genes and DNAs rather than phenotypic and characteristic complementary between parent lines (Breseghello and Coelho, 2013; Miah et al., 2013). Generation of the first *Agrobacterium* introduced transgenic plants marked the beginning of plant molecular breeding era and the molecular marker system was developed soon afterwards (Bewan et al., 1983; Edwards et al., 1987; Herreraestrella et al., 1983; Rafalski and Tingey, 1993). Nowadays, with the global dissemination and application of molecular plant breeding techniques, many approaches have been developed,

including QTL (Quantitative Trait Loci) mapping or gene discovery, MAS (Maker Assisted Selection), genomic selection, genetic engineering and genetic transformation (Breseghello and Coelho, 2013; Collard et al., 2005; Moose and Mumm, 2008).

Marker Assisted Selection (MAS) is a powerful tool, especially during the selection of one gene or several genes, controlling simple traits such as disease resistance and typical qualitative traits (Norman and Norman, 2008). Once target genes closely linked markers were identified, plants with those specific genes or QTLs can be quickly identified through DNA marker alleles prior to large-scale field examination (Collard et al., 2005; Michelmore, 1995; Young, 1996). The implementation of resistance gene pyramiding, which is almost impossible with phenotypic-dependent conventional breeding, relies strongly on MAS, (Breseghello and Coelho, 2013; Collard et al., 2005). By accumulating several resistance genes into one individual, it would be difficult for a pathogen to beat all genes simultaneously and this leads to more durable resistance and slows down the process of resistance breakdown (Hittalmani et al., 2000). A large amount of DNA markers or molecular markers have been explored as tools in molecular plant breeding including: random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RPLPs), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs) or microsatellites and single nucleotide polymorphisms (SNPs) (Botstein et al.,1980; Hearne et al., 1992; Vos et al., 1995; Welsh and Mcclelland 1990; Williams et al., 1990).

Most agriculturally important traits, including yield, plant height and some disease and pest resistances, are quantitative traits, under the control of multiple genes (Collard et al., 2005; Madan et al., 1997). Therefore, a QTL mapping approach is of great value, especially in the identification of genetic properties of quantitative traits (QTLs), which illustrates the amount of genes in charge of certain quantitative trait, the relative importance or the contribution of multiple genes and their

location in the genome (Breseghello and Coelho, 2013; Price, 2006; Von Brothmer, 2003). Many valuable quantitative traits in various crops have been discovered through the combination between QTL mapping and MAS, including *Fusarium* head blight resistance in wheat (Bourdoncle and Ohm, 2003); GW5, a QTL in charging of grain shape and biomass in rice species (Weng et al., 2008) as well as Ghd 8, the major QTL associated with grain production in rice (Yan et al., 2001).

Genetic transformation and engineering is a relatively novel and controversial scientific field used in plants, animals and microorganisms. Gene transformation enables gene exchanges between various species, providing boundless genetic resources and opportunities in future breeding (Gasser and Fraley, 1989; Kbrs et al., 2009). Depending on the medium used, there are two classifications: 1. vector-mediated methods including the *Agrobacterium*-mediated transformation using bacteria as vector (Schell and Montagu, 1977) or viral transformation in which plant virus acts as transformation medium (Petrovsky and Nikolai, 2012); or 2. vector less strategies, mainly characterized by the use of gene gun (Décima et al., 2010; Klein and Jones, 1999) and electroporation (Zhou et al., 1993). Even though highly controversal, genetic engineering is still thought to be a promising tool and has produced a few commercialized genetically modified (GM) crops including maize, soybean, and potato.

2.4 Classification of mycorrhizae and arbuscular mycorrhiza (AM)

2.4.1 Crop improvement through beneficial plant-microbe interaction

Based on the different types of interactions between plant hosts and microorganisms, microbes are broadly divided into two groups: pathogens, affecting hosts negatively, and plant beneficial microbes, with which a establish a mutually beneficial association with the plant. The latter one being promising in the development and improvement of modern agriculture. Diversity of

microbes, such as bacteria and fungi, have been proven to form beneficial symbiosis with plant root systems. Among them, rhizobia and mycorrhizal fungi are two of the most researched microbe groups; being paid extremely high attention to by numerous researchers (Lahrach et al., 2013; Jeffries and Rhodes, 2008; Oldroyd et al., 2010).

Rhizobia are a group of highly specialized bacteria which establish mutualistic association with legumes and *Parasponia*. It contains a wide range of bacterial genera, represented by *Rhizobium*, a widely applied bacteria genus (Sessitsch et al., 2002). By colonizing root nodules of host plants (mainly leguminous plants) rhizobia fix atmospheric nitrogen and convert it into ammonia, the material used for further synthesis of organic nitrogen in the shape of glutamine or ureides. In return, the host provides energy recourses to support the bacteria (Hiroyuchi et al. 2003; Press and Poole, 2006; Udvardi and Poole, 2013). Even though a large amount of research has been conducted to further understand and to expand utilization of this mutualistic symbiosis, the high specialization of rhizobial bacteria limits its development and application. Therefore, for wheat, more attention would be paid to mycorrhizae.

Mycorrhizae are ancient intergrowth structures formed between plant roots and mycorrhizal fungi (Kirk et al., 2001; Sawers et al., 2008). Compared with Rhizobia, mycorrizae have significant advantages, colonizing a much wider range of hosts (over 80% of existing plants) and benefiting hosts in multiple aspects, ranging from enhanced nutrient absorption and transpotation to improved tolerance against diverse stresses (Cameron et al., 2013; Fitter, 1997; Pozo and Azcón-Aguilar, 2007; Samers et al., 2008).

2.4.2 Mycorrhiza and its classification

A mycorrhiza is a root-specific symbiosis structure formed between fungi and the majority of terrestrial plants when fungi colonize on the hosts' roots (Kirk et al., 2001; Sawers et al., 2008).

As concluded by Wang and Qiu (2006) mycorrhizal symbiosis, especially composed with arbuscular mycorrhizal fungi (AMF), have be found in over four fifths of the documented plant species and ninety percent of the studied plant families (Wang and Qiu, 2006). Host plants take advantage of this symbiotic system in various means. For example, water uptake and water content inside host plants is promoted, mineral nutrient absorption (especially phosphorous) is enhanced and stress tolerances are strengthened in plants such as wheat (Al-Karaki and Clark, 1998; Hetrick et al., 2011; Mustafa et al., 2013), maize (Murdoch et al., 1967; Sheng et al., 2008) as well as pepper (Salami, 2002; Turkmen et al., 2008) due to the formation of mycorrhizae. There are, however, also some expectations in which mycorrhizal fungi are pathogenic to hosts.

Generally, there are two types of mycorrhizae, ectomycorrhiza and endomycorrhiza; differentiated by how fungal hyphae interact with root cells. Ectomycorrhizas occur on approximately 10% of plant families (mainly woody plants), and are characterized by fungal hyphal elongation between root cells but without penetrating individual cells (Allen, 1992; Wang and Qiu, 2006). Endomycorrhizae, especially arbuscular endomycorrhizae (the most common type), ericoid and orchid mycorrhizas, are formed when fungal hyphae penetrate root cells and invade into cell membranes (Allen, 1992; Barber et al., 1977).

2.4.3 Arbuscular Mycorrhiza (AM)

Arbuscular mycorrhiza (AM), previously known as vesicular-arbuscular mycorrhiza (VAM), are the most prevalent and predominant endomycorrhiza, and are formed between Glomeromycota fungus and 85% of studies plant families, ranging from angiosperms (especially many crop species), pteridophytes to mosses and lycopods (Arthur et al., 2001; Harley and Smith, 1997; Wang and Qiu, 2006). The fungus grows in and penetrates root cells, where it forms the branching invaginations named arbuscules after which the mycorrhiza was named (Harley and Smith, 1997).

It has been proven through DNA sequencing and fossil analyses that arbuscular mycorrhiza is one of the oldest mutualistic associations (probably the ancestor of endomycorrhiza) and it has been 400~460 million years since its first appearance, which is concurrent with the emergence of earliest terrestrial plants (Parniske 2008; Remy et al., 1994; Redecker et al., 2000; Simon et al., 1993). Arbuscular mycorrhiza play a significant important role in modern agriculture especially when increasing controversy appears on overuse of chemical in agriculture.

2.5 Mineral absorption by arbuscular mycorrhizal (AM) association

Material exchanges occur within the AM mutualistic symbioses. Host plants provide *Glomeromycota* with photosynthetic carbohydrates such as glucose and sucrose to support fungal life (Bago et al., 2000; Harrison, 2005; Harley and Smith, 2006). In return, fungi uptake and transfer soil-derived mineral nutrients, especially phosphorus and water, to the roots, altering the mineral content and water status in host plants (Harley and Smith, 2006; Marc-André, 2006). It has been previously reported by Marschner and Dell that AM associations benefit plants through increasing uptake of multiple mineral nutrients, especially P, N, K, Zn and Cu by 80%, 25%, 10%, 25% and 60%, respectively (Marschner and Dell 1994). Statically, plants generally convey 4% to 20% of their total photosynthetic carbon to support the mycorrhizal association, with an annual amount reaching 5 billion toones (Douds et al., 2000).

Various mechanisms are discovered to be responsible for the increased absorption of water and mineral nutrients and it could be generally explained from three aspects: physics, chemistry and biology (Mahanta et al., 2014). With the colonization of AMF, a well-developed hyphae network is shaped around the roots, positively enhancing mineral uptake. Taking the absorption and transfer of phosphorus as an example: the established hyphae network elongates the root and enables the uptake of nutrients from several centimeters away. Moreover, since mycelium has significantly

lower diameters compared to roots, it allows hyphae to access to smaller pores in soil, increasing the volume of soil hyphae exposed and raising surface area for a better and more direct phosphorous absorption (Smith et al., 2011). Additionally, previous research has also found that a complicated hyphael networks could be built between adjacent plants; which creates the possibility of a broader material exchange between individuals (Simard et al., 2012). Finally, polyphosphates also play a fatal role in mineral uptake and transfer within the association. Behaving as a carrier for cations like magnesium, potassium and amino acids due to its strong negatively polyanions, polyphosphates benefit plants not only by accelerating transportation of phosphorus but also promoting the acquisition of mineral nutrients other than P (Marschner, 1997). The differences in the chemical characteristics of mycorrhizal fungi and plant roots also contributes to strengthened nutrient uptake. Plant roots are unable to acquire demineralized phosphate ions when soil conditions are basic, however fungal hyphae make it possible by excreting H^+ ions and creating an acidic environment around the rhizosphere, which makes phosphorus ions more soluble (Hamel, 2004; Li et al., 2006). Additionally, the switch between NH_3 and HH_4^+ releases H^+ to root surroundings and provides nitrogen flow to plant hosts simultaneously. Further physiological and molecular researches on crops such as rice, wheat, sorghum and barley have revealed that Pi transporters play a significant role in phosphorus uptake (Donna et al., 2005; Javot et al., 2007; Walder et al., 2016). To be specific, inoculation of AMF induces the expression of PiT gene in root cortical cells and produces a specific Pi transporter which collaborates with other Pi transporters to facilitate the absorption of phosphorus (Smith et al., 2011; Walder et al., 2016).

Many internal and external factors have been proven to affect mycorrhization induced nutrient absorption, especially the effects of carbon provision. Bücking and Shachar-Hill have reported the that supplement of photosynthetic carbohydrates from plant green tissues (such as leaves) to

mycorrhizal fungi is positively correlated to the acquisition and transfer of phosphorus from mycorrhizal association to plants (Black et al., 2000; Bücking and Shachar-Hill, 2005). Moreover, abundant research has demonstrated that external environmental stresses and the inherited differences are important elements to consider when evaluating efficiency of nutrient absorption (Bethlenfalvay et al., 1988; Smith et al., 2003).

2.6 Salinity resistance of mycorrhizal plants

2.6.1 Detrimental salinity effects on host plants and mycorrhizal fungi

Soil salinization is an increasingly severe problem existed globally and is particularly worse on arid and semi-arid regions (Giri et al., 2003; Al-Karaki, 2006). Currently, approximately 77 million hectares (over 5%) of global cultivated farmlands are heavily eroded or even degraded by salt-alkalization and by the middle of 21st century, the percentage of land-loss resulting from soil salinization is estimated to reach up to 50%, along with devastating productivity losses in agriculture and economy (Giri et al., 2003; Munn et al., 1999; Wang et al., 2003).

Salinization provides plants with a detrimentally environmental condition and therefore negatively impedes plants' growth vigor and developmental process from three main aspects (Bernstein, 1975; Evelin et al., 2009; Parida and Das, 2005; Porcel et al., 2012). First, reduced osmotic potential in saline soil makes it difficult for plants to uptake water from their surroundings, leading to physiological drought. Plants have to struggle to lower intercellular osmotic potential to maintain intercellular water content and to absorb soil water from habitat (Feng et al., 2002; Jahromi et al., 2008; Munns and Termaat, 1986). Additionally, salinity triggers nutrient imbalances, which mainly results from superfluous absorption and transfer of sodium and chlorine ions but can result in deficiencies in other ions (Abel and Matthes, 2001; Marschener, 1995). Moreover, it has been discovered that excessive sodium and chloride ions from soil are toxic to plants, disrupting

enzymes structure and relevant metabolic reactions and through damage of cell organelles (Feng et al., 2002; Juniper and Abbott, 1993; Netondo et al., 2004).

Salinity stress behaves detrimentally not only to host plants but also to mycorrhizal fungi, especially in lowered germination rate of fungal sporulation (Estaun, 1990; Hirrel, 1981), poorly developed fungal hyphae (Estaun, 1990; Mcmillen et al., 1998) and reduced colonization capacity (Nourmandipour et al., 2014; Peroza and Perez, 2010). It has previously been reported that salt delays spore germination through altering the amount, types and concentration of saline ions within fungal cells (Juniper and Abbott, 2006). This was supported by Juniper and Abbott's findings (1993) where they found that: 1) A faster germination rate and higher maximum germination were detected in NaCl solution than in KCl solution with similar osmotic potentials; and 2) Even with same ion and similar osmotic potentials, less spores germinated when treated with Na_2SO_4 than with $NaNO_3$, because of a higher concentration of Na^+ in the former (Juniper and Abbott, 1993). Elongation of hyphae responded negatively to salinity treatment in in vivo studies, and the reduction on hyphal development in turn hamper fungal colonization in roots and hinder the formation of mycorrhiza (Jahromi et al., 2008; Juniper and Abbott, 2006). Generally, fungal colonization rate decreased in the present of salt, as founded in maize (Sheng et al., 2008), onion (Ojala et al., 1983) and tomato (Al-Karaki et al., 2001; Latef et al., 2011). One possibility is the saline-caused direct changes in fungi, such as shorter and less numerous fungal hyphae (Jahromi et al., 2008). Inherited difference between mycorrhizal fungal species and environmental conditions are also potential factors for altered colonization patterns (Carvalho et al., 2004; Klironomos et al., 1993). There are however, some exceptions showing that the colonization rate of mycorrhizal fungi is not changed or even increase under salt stress, important findings in the search for salt-resistant AMF species (Aliasgharzadeh et al., 2001; Levy et al., 1983).

2.6.2 Mechanisms for mycorrhizal induced salinity resistance

Plenty of previous studies have found that plants take advantages from mycorrhiza under salt stress as compared to non-inoculated plants, with stronger absorption capacity for mineral nutrients and available water from saline soil, more intracellular osmotic regulation compounds and antioxidants, higher photosynthesis rate and better water use efficiency. Those phenomena indicate that mycorhiza induced salinity resistance is the combination of multiples factors, involving nutrient uptake, biochemistry reactions and physiology effects (Evelin et al., 2009; Çekic et al., 2012; Jahromi et al., 2008; Porcel et al., 2012) with types and degrees of the benefits varying between selected AMF species (Gong et al., 2012; Marulanda et al., 2007; Wu et al., 2007; Zou and Wu, 2011).

Selective enhancement of mineral nutrient uptake by mycorrhizal association is one response mechanism for salinity tolerance. Mycorrhizal colonization has long been known to improve nutrient uptake in both normal and saline soils, especially the immobile P, which facilitates hosts' growth and development (Al-Karaki, 2000; Mardukhi et al., 2011; Red et al., 2009; Talaat and Shawky, 2013). The enhanced absorption of selected mineral ions plays a role in osmoregulation and can act protectively in saline soils. Taking potassium ions as an example; mycorrhizal association increases the acquisition of K^+ for a higher K^+/Na^+ ratios when exposed to highly Na^+ saline soil. This was previously reported in arbuscular mycorhiza inoculated *Acacia nilotica*, tomato, wheat and barley (Abdel-Fattah and Asrar, 2012; Giri et al., 2007; Latef et al., 2011; Mohammad et al., 2003; Munir et al., 2003; Talaat and Shawky, 2011; Talaat and Shawky, 2013). This alternation helps lower the intercellular osmotic potentials with increased amount of K^+ in cells to improve acquisition of water and other minerals such as phosphorus, copper and zinc; it

also prevents metabolic processes and protein synthesis from being disrupted by Na^+ (Al-Karaki and Clark, 1998; Mark and Romola, 2003; Talaat and Shawky, 2013).

Osmotic regulation can be achieved not only through selective absorption of mineral ions like K+ and Na+, but also through accumulation of alternative organic molecules as additional sources of osmoregulation substances, which includes amino acid proline, glycine betaine, soluble carbohydrates, etc. (Feng et al., 2002; Flower and Colmer, 2008; Morgan, 2003; Rabie and Almadini, 2005). Increased amounts of osmolytes are accumulated in root cells when plants are inoculated by mycorrhizal fungi; this decreases the intercellular osmotic potential, which helps plants maintain water, and protects organelles and metabolic enzymes (Bohnert and Jensen, 1996; Ferraris et al., 1987; Leffler, 1993).

It has been proven in previous studies that abiotic stresses such as salinity induce oxidant stress, as the consequence of ROS (Reactive Oxygen Species) accumulation resulting from degeneration (Gossett et al., 1994; Gottlied et al., 2009; Hajiboland and Joudmand, 2009; Hernández et al., 1994; Lei et al., 2005). However, mycorrhizal symbiosis helps plants alleviate the poisonous effects from salinity stress/oxidant stress through strengthening activity of antioxidant enzymes and synthesizing increased numbers of antioxidants (Alguacil et al., 2003; Talaat and Shawky, 2011). This prevents functional molecules like DNA, proteins and lipids from oxidant damages and thereby maintains regular cellular activities (Bowler et al., 1992; Flynn et al., 1983). Abundant evidences have been found to prove that plant mycorrhization is positively correlated with antioxidant capacity by enhancing activity of anti-oxidase enzymes in diverse plant species, ranging from shrub (Alguacil et al., 2003) to crops like wheat (Talaat and Shawky, 2011) and soybean (Ghorbanli et al., 2004). Measurement of total phenolic content (TPC) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging capacity are two major ways in the evaluation of antioxidant

capacity, correlating either positively or negatively. The quantification of TPC, components with antioxidant capacity, reflects the antioxidant activity of plants, in which the higher TPC values are, the stronger antioxidant capacity plants possess (Kähkönen et al., 1999). DPPH scavenging capacity assay, however, evaluates antioxidant capacity of plants by measuring the elimination efficiency of detrimental oxidant radicals and it is widely proved that the more DPPH free radical scavenged, the stronger antioxidant capacity is (Braca et al., 2002).

Mycorrhizal colonization further benefits plants through alleviating salt-induced physiological damages, reflecting on promoted recovery of photosynthetic efficiency, maintenance of membrane integrity and enhanced water status and gas exchange rate (Evelin et al., 2009; Jahromi et al., 2008; Porcel et al., 2012; Sheng et al., 2008). To be specific, a higher photosynthetic efficiency, which is estimated by higher chlorophyll concentration, is broadly observed in mycorrhizal inoculated plants than in non-inoculated plants in alkali soil, which partially results from the increased absorption of magnesium ions, the key mineral ion in chlorophyll synthesis (Datta and Kulkarni, 2014; Kumar et al., 2010; Murkute et al., 2006; Sannazzaro et al., 2006; Sheng et al., 2008; Wu et al., 2015). Salt-induced photosynthetic efficiency decrease is also recoverable through protection of the PSII reaction center from being disrupted by saline toxicity and through improvement of the non-photochemical quenching to avoid light damage to leaves (Sheng et al., 2008; Maxwell and Johnson, 2000). Secondly, abiotic stresses like salinity and drought could cause damages to plants by disrupting membrane integrity. Mycorrhizal colonization, however, enables to relief damages through maintaining the integrity and stability of cellular membrane, which is mainly attributable to the increased P absorption and improved antioxidant protection (Beltrano et al., 2013; Feng et al., 2002; Kaya et al., 2009; Zheng et al., 2011). In addition, regulation of water status within mycorrhizal symbiosis is also of great importance in salinity tolerance. Generally, one main

negative effect caused by salinity stress is physiological drought, which makes it hard for plants to uptake water from a plant's surroundings in highly saline soil (Anna et al., 2008; Evelin et al., 2009; Feng et al., 2002; Jahromi et al., 2008; Porcel et al., 2012). However, the formulation of mycorrhizal association relives physiological drought effect mainly through increasing water content and up-regulating water use efficiency. A comparison between mycorrhizal inoculated and non-inoculated plants reveals that water content significantly increases after plant mycorrhization. This increased water content has been universally reported in previous researches and probably attributes to the comprehensive effects of the root morphology alternation, turgor potential elevation, hydraulic conductivity improvement as well as water saturation reduction (Al-Garni, 2006; Colla et al., 2007; Dehne, 1982; Kapoor et al., 2008; Lerner et al., 1994; Sheng et al., 2008; Yoseph et al., 1983). The convergence of these factors also results in a more efficient usage of intercellular water and therefore a lowered intercellular concentration of carbon dioxide (Evelin et al., 2009). Finally, gas exchanges are in turn facilitated as the result of low carbon dioxide, along with the regulation of ABA hormone and ABA/cytokinin equilibrium, to relieve saline damages (Graham and Syvertsen, 1984; Goicoechea et al., 1997; Jahromi et al., 2008; Sannazzaro et al., 2007).

2.7 Transposon as tools for functional genomics

2.7.1 Transposon and its classification

Transposons or transposable elements (TEs), originally named as "jumping genes", are DNA fragments that are able to alter their locations within genomes by transferring from original sites to new positions and therefore creates mutants (Martín and García-Pérez, 2010; Mcclintock, 1950). It is a representative family of the mobile genetic elements (MGE). The first TE was discovered by Barbara McClintock in maize in 1949 which lead her to receive the Nobel prize in 1983 for this

earthshaking discovery (Mcclintock, 1950; Read, 1993). Transposons are prevalent in both prokaryotes such as bacteria (Martiel and Blot, 2002) and eukaryotes including corns (Gierl et al., 1989), yeasts (Williamson, 2011), fruitflies and humans (Nekrutenko and Li, 2001) and make up a large part of genomes. As reported in previous studies, more than 90% of the maize genome and 40% of human genome are composed of TEs (Sanmiguel et al., 1996; Mills et al., 2007). Generally, transposons are broadly divide into two classes depending on the transposition mechanisms: the RNA-intermediated retrotransposons and the DNA-intermediated transposons (Capy et al., 1996; Feschotte and Pritham, 2007; Kumar and Bennetzen, 1999; Liu et al., 2002; Wicker et al., 2007).

Class I: RNA-intermediated Retrotransposons

The retrotransposon, a RNA-intermediated TE family is uniquitous in eukaryotic cells. 50-78% of the maize genome and 42% of human genome is made up of retrotransposon (Cordaux and Battzer, 2009; Lander et al., 2001; Sanmiguel and Beenetzen, 1998). The retrotransposons function with a "copy and paste" mechanism, in which the original transposable DNA fragment is first transcribed into RNA before reverse transcribing to DNA under the regulation of reverse transcriptase and integrating into new position of the genomes (Boeke and Chapman, 1991; Dombroski et al., 1994; Fink et al., 1986). As a consequence, the retrotransposition explosively enlarges the genome sizes including new stable mutants (Fink et al., 1986; Vicient et al., 1999).

Retrotransposons consist of two subgroups, distinguished by difference in structure and reverse transcriptase: 1) long terminal repeat (LTR) retrotransposons; 2) non-LTR retrotransposons (Adams et al., 1987; Doolittle and Mcclure, 1989; Finnegan, 2012; Xiong and Eickbush, 1990). The LTR retrotransposon is characterised by its retroviruses-like long terminal repeat (LTR), which qualifies it structural and functional similarity with retroviruses (Finnegan, 2012; Potter 2005). The non-LTR retrotransposon is characterised by long or short interspersed sequences

instead of LTR and there are consequently two main subgroups in non-LTR retrotransposons classified by properties of interspersed sequences (Grandi and An, 2013; Han 2010). Long interspersed nuclear element (LINEs) is an autonomous transposable element, which encodes reverse transcriptase and transposes themselves, while the short interspersed nuclear element (SINEs) is a non-autonomous transposable element that possesses no reading frames and therefore disables to mediate its own transposition (Cheng and Liu, 2006; Das et al., 1998; Geier et al., 1996; Konkel et al., 2010; Noma et al., 1999; Torsten et al., 2011).

Class II: DNA transposons

Different from retrotransposons, transpositions of DNA transposons depend on DNA-intermediates. Compared with retrotransposons that make up over 40% of human genome, DNA transposons are less ubiquitous, comprising only 2% of human genome (Richard and Batzer, 2009; Lander et al., 2001). The DNA transposons abide generally by a "cut and paste" mechanism as represented by the corn *Ac*/*Ds* transposons, in which a stagger cut is made at the target site to generate "sticky ends" before the served fragment is transferred and ligated into new location with genome (Feschotte and Pritham, 2007; Martín and García-Pérez, 2010). In this way, the transposition activities affect only within the original and new locations of TEs but have little effects on whole genome size (Kunze and Starlinger, 1989).

2.7.2 Transposon as a genetic tool

The transposon system is a widely used approach in random insertional mutagenesis for gene function analysis in various plant species such as maize, rice, barley, tomato, *Arabidopsis*, etc. (Aarts et al., 1993; Bishop et al., 1996; Ishida et al., 1996; Izawa et al., 1997; Mccarty et al., 2005; Miyao and Hirochika, 2004; Singh et al., 2012). Compared with other insertional mutagenesis approaches such as T-DNA mutagenesis, transposon mutagenesis has significant advantages. First,

transposon based insertion units are usually intact elements without undesirable tandem repeats or deletion, making it easier to molecular identify and subsequent sequence analysis (Ramachandran and Sundaresan, 2001). Secondly, the characteristic of transposons enables the insertion fragment to be excised, which makes phenotypic reversion available (Frøkjaerjensen et al., 2010; Haniford, 2001). Moreover, the preference of transposons to insert into genetically linked locus enables local mutagenesis (Brown et al., 2015; Ito et al., 1999; Jones et al., 1990; Seki et al., 1999; Singh et al., 2016; Singh et al., 2012;). Furthermore, discovery of two-component systems, represented by such *Ac*/*Ds* or *Spm*/*dSpm*, increases the controllability and stability of transposon mutagenesis. Finally, transposon-based insertion generates both loss-of-function mutants (caused by gene disruption) and increased gene expression (through activation tagging) (Ayliffe et al., 2007; Tani et al., 2004).

2.7.3 *Activator*/*Dissociation* (*Ac*/*Ds*) transposons

The maize *Ac*/*Ds* transposable element system was first discovered by Barbara McClintock in 1940s (McClintock, 1947; McClintock, 1948) and lead her won a Nobel Prize in 1983. At the same year, Federoff and his team successfully isolated *Ac* and *Ds* elements from inserted maize Waxy 1 gene and sequenced for the first time (Fedoroff et al., 1983). Involved in the DNA-intermediated transposons, transposition of maize *Ac*/*Ds* follows the "cut and paste" mechanism. Specifically, the *Ac* element (activator) encodes a transposase, which enables *Ac* to transpose autonomously. However, because the absence of self-encoded transposase, the *Ds* element (dissociation) is unable to transpose autonomously. *Ds* transposition relies on the transposase provided by *Ac* transposable element, which makes *Ds* transposition controllable (Du et al., 2011; Gorbunova and Levy, 1997; Kunze and Starlinger, 1989). Both *Ac* and *Ds* transposable elements contain a 11 bp terminal inverted sequence followed by a 250 bp sub-terminal regions on both ends of the TEs, which is crucial for the transposition activities (Coupland et al., 1998; Varagona and Wessler, 1990).

Tobacco (*Nicotiana tobaccum*) was the first heterologous plant in which the maize *Ac*/*Ds* was introduced into and applied successfully (Baker et al. 1987; Baker et al., 1986). Since then, the *Ac*/*Ds* transposons have been broadly used as a functional genomic tool in various plants, including rice (Rengasamy and Ramamoorthy, 2009; Solis et al., 1999; Yu et al., 2012), barley (Ayliffe et al., 2007; Singh et al., 2006; Singh et al., 2011), tomato (Biezen et al., 1996; Carter et al., 2013), lettuce (Michelmore, 1994; Yang et al., 1993), etc.

These two-component transposons possess significant advantages over single component transposon and one of the most remarkable superiorities is the controllability of the *Ds* transposition. The segregation of transposase gene enables the stabilization and adjustability of transposition activities of the non-autonomous *Ds* transposon (Fedoroff 1989; Hehl and Baker, 1989). Moreover, the *Ac/Ds* transposon system realizes the phenotypic reversion through *Ds* excision, which could be easily activated with the present of *Ac* transposase (Lazarow et al., 2013; Lida et al., 1982). Up to now, numerous genes have been discovered through *Ac/Ds* transposons system, including the anther indehiscence1 gene in rice (Zhu et al., 2004), *Curly leaf* in Arabidopsis (Goodrich et al., 1997), *knotted 1* in corn (Hake et al., 1989), etc.

Connecting Text

The following chapter evaluates the response of spring wheat cultivars to arbuscular mycorrhizal (AM) colonization under salinity stresses. Arbuscular mycorrhizae have proven to be beneficial to diverse plants such as rice, barley, corn, and wheat, especially under stress conditions like drought and salinity. Based on the literature review, arbuscular mycorrhizal inoculation is one of the most promising approaches for the alleviation of stress-induced damages. However, benefits vary between mycorrhizal strains and host species, which emphasizes the importance of arbuscular mycorrhizal strains selection. In the research described below, we selected four spring wheat cultivars and four mycorrhizal strains, treated with three salinity levels and variables including plant biomass and root morphology were measured for comparison. These findings have been submitted to the Journal of Pedosphere for review.

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Chapter 3

The response of spring wheat cultivars to arbuscular mycorrhiza (AM) colonization under salinity stresses

3.1 Abstract

Wheat is an important crop, playing inevitable roles in every aspect of human life, ranging from major food resource and prefect antioxidant donors to raw materials for biofuel. However, wheat production nowadays is undergoing extreme challenges as a consequence of the dramatically reduced available arable areas and increasingly severe abiotic and biotic stresses within habitat. Many approaches have been explored to alleviate wheat yield loss, such as discovery of novel stress tolerance cultivars, soil condition improvement and alternation of cultivation systems, etc. One of the most promising approaches is the application of the naturally existent arbuscular mycorrhiza (AM), a mutualistic symbiosis originated over 400 million years ago. Arbuscular mycorrhizae have long been known to form mutualistic symbiosis with various plants to enhance hosts' yield production and to improve plants' stress tolerance, especially drought and salinity. But the benefits vary among AM strains and plant species. Therefore, the objective of the study was to investigate the influence of four AM strains colonized on four selected spring wheat varieties under three salt concentrations (0, 50, 100 mmol/L). Data on plant biomass and root morphologic variables were measured and analyzed with SAS PROC GLIMMIX (Generalized Linear Mixed Models). The results demonstrated that wheat inoculated with arbuscular mycorrhizal strains *Funneliformis mosseae* and *Rhizophagus irregularis* mitigated yield losses caused by increased salinity stresses as well as strengthened root growth in comparison with non-inoculated plant controls. Salinity stress, however, had non-significant negative effects on most variables, except

for grain yield, root surface area and root dry weight, in which a significant decrease was observed in root surface area and root dry weight with the increasing of saline concentration.

3.2 Introduction

The importance of wheat (*Triticum aestivum L.*) cannot be disputed, as it ranks among the top cereal crops and is grown globally. In 2013, with a global production over 0.71 billion tons, wheat ranked as the third most produced crops, following after 0.74 billion tons of rice and more than 1 billion tons of maize (FAOSTAT 2013). Planted on more than 17% of global crop acreage, it successfully feeds over two-fifths of population worldwide (Gupta et al., 2008). Today, however, numerous severe stresses, both biotic and abiotic, impede the global wheat production and soil salinization is one of the most severe concerns, with more than 77 million hectares of cultivated farmland eroded by high salinity globally (Munns et al., 1999). Many approaches have been applied to alleviate yield loss caused by soil salinization, including exploration of novel stress tolerance cultivars through modern breeding methods (Mozafar and Goodin, 1986), optimization of cultivation systems to reduce negative effects from salt salinization (Sun et al., 2010) as well as the application of naturally pre-existed beneficial symbiosis (Beltrano and Ronco, 2008). One most promising way is the exploration and usage of arbuscular mycorrhizae, a mutualism symbiosis that would protect plants from saline damages.

With a history of more than 400 million years, arbuscular mycorrhizas are considered to be one of the oldest and most prevalent symbiosis formed between plants and fungi species in the world (Parniske 2008; Pirozynski and Malloch, 1975; Remy et al., 1994; Simon et al., 1993). It has been reported in previous studies that more than 85% of documented plant species are putative hosts of arbuscular mycorrhizal fungi and they take advantages from the symbiosis in various aspects, ranging from increased water and mineral nutrient absorption, especially P, the crucial mineral for

plant growth and development, to improved biotic and abiotic tolerances through multiple complex mechanisms (Daei et al., 2009; Harley and Smith 1997; Marschner and Dell 1994; Pozo et al., 2010; Wang and Qiu 2006). The increase in salinity tolerances have been observed in numerous mycorrhizal colonized plants, represented by crops like barley and wheat (Rad et al., 2009; Tallat and Shawky 2014). However, these benefits vary between AM strains selected and host plants involved (Gong et al., 2012; Zou and Wu, 2011). Therefore, the objective of this project was to evaluate the response of four selected spring wheat genotypes to the inoculation of four arbuscular mycorrhizal strains under three salt concentrations.

3.3 Material and Methods

3.3.1 Experimental material

---- Wheat varieties

Four spring wheat cultivars, namely FL62R1, Scotia, Snowbird and 13NQW1265, were analyzed in the current study (Table 3.1). In addition to plant biomass and root morphology discussed in this experiment, the relationship between grain color and antioxidant components and their responses to wheat mycorrhization were also of great interest. Therefore, four spring wheat varieties were chosen and used in our study, mainly considering their representative grain colors (red, white and purple grain). One one hand, alternations of plant biomass and root morphology were measured and analyzed and the results were discussed in this study. On the other hand, the harvested grains would be further processed for grain antioxidant components analysis for the understanding of differences in antioxidant capacity of color wheat when inoculated and non-inoculated by AMF strains, as reporter in Chapter 5 below.

----Arbuscular mycorrhizal strains

Four typical arbuscular mycorrhizal strains *Funneliformis mosseae* (*F. mosseae*), *Funneliformis caledonius* (*F. caledonius*), *Rhizophagus irregularis* (*R. irregularis*) and the commercial strain *Myke* (*R. irregularis*) were used, which enabled the comparison of interspecific and intraspecific strain potential. The arbuscular mycorrhizal strains were provided by Dr. Yolande Dalpé from Ottawa Research and Development Centre, Agriculture and Agri-Food Canada.

F. mosseae, usually isolated from wheat crop cultures, is a world-wide distributed AMF species and currently found in cultivated soils in temperate climate countries. While *F. caledonius*, currently in temperate climate countries, is mostly found in non-cultivated lands (indigenous soils) but can be quite efficient on symbiosis establishment sometimes.

R. irregularis strains are generalist strains currently used in commercial products. *R. irregularis* (DAOM 240442) comes from the Canadian national collection of *Glomeromycota* and the *Myke* is the most common strain used by inoculant companies in the world. These two AMF strains originate from the same mycorrhizal species but different strains and therefore it is possible to compare intraspecific strain potential by involving these two species.

----Salinity treatments

Three salinity levels (0, 50, or 100 mmol/L) were used and saline solutions were made with sodium chloride (NaCl).

3.3.2 Methodology

A greenhouse experiment was conducted under regulated condition with a 16-hour photoperiod per day and 22°C /20°C (day/night) temperature regime. Four selected spring wheat cultivars, four arbuscular mycorrhizal strains and three salinity levels (0, 50, or 100 mmol/L) were used. For each wheat variety, five seeds inoculated with one mycorrhizal strain at a rate of 50 spores was planted

in each 6″ pot, with equal distance between each seed, along with non-inoculated controls. Inoculants were provided in form of specific-spore-containing matrix with the concentration of spores detected. Non-mycorrhizal controls were treated with mycorrhizal-free AGRO MIX G10 soil instead of mycorrhizal-spore-containing matrix. 360 pots were used with 60 pots per replication and 6 replications in total, setting up following the randomized complete block design (RCBD). The AGRO MIX G10 soil (AF) was used as soil matrix in this experiment, which guaranteed no extra mycorrhizal fungi in soil. Constant irrigation was provided throughout whole growth period but stopped on the exact days when saline treatments were applied. Plants were fertilized (a fertilizer named 20-20-20) every two weeks with 100 ml per pot with a concentration of 1% (1 gram of fertilizer 20-20-20 into 1 liter of water).

Salinity treatment with 100 mL of NaCl solution (0, 50, or 100 mmol/L) per pot initiated since the fifth week at a three-day interval and lasted for six weeks until harvest. Data on grain yield, root length, root surface area, root total volume, fresh root weight and dry root weight were collected and measured. Root morphologic parameters were collected with *WinRHIZO Pro* image analysis software and root dry biomass was then measured after drying in oven at 60°C for three days. Data were analyzed using SAS PROC GLIMMIX (Generalized Linear Mixed Models), considering the diverse distributions of data. Instead of transferring all data into uniform natural distribution format, statistic analysis was conducted based on the original distributions of variables.

3.4 Results

3.4.1 Plant Biomass (grain yield, root fresh weight and root dry weight)

----Grain yield

Grain yield, root fresh weight and root dry weight were measured and analyzed as indictors of

plant biomass. With a *p*-value of 0.0121, grain yield was significantly affected by the interaction between mycorrhizal strains and salinity treatments (Table 3.2). Wheat varieties, however, had no significant effects on grain yield as per the statistical results (*P*=0.0869). Specifically, salinity levels had a significant negative effect on yield production within non-mycorrhizal controls (*p*-value of 0.0205) and *Funneliformis caledonius* inoculated wheat cultivars (*p*-value of 0.0027), representing as a significant yield decrease when treated with 100 mmol/L NaCl solution as compared to non-salt controls. There were, however, no significant fluctuations on grain production between salt and non-salt treatments when wheat was inoculated with other arbuscular mycorrhizal fungi strains, indicating that the colonization of AMF strains *Funneliformis mosseae*, *Rhizophagus irregularis* (DAOM240442) and *Myke* (DAOM197198) benefited host wheat by efficiently mitigating saline-induced yield decrease.

----Root fresh weight

Root fresh weight was significantly influenced by wheat-mycorrhiza interaction with a *p*-value around 0.0137 (Table 3.3). Salt treatment, however, had no significant effects on root fresh weight (*p*-value 0.0548). To be specific, wheat Snowbird and wheat 13NQW1265 responded positively to the inoculation of fungi strain *Rhizophagus irregularis* (DAOM240442), resulting in a significant heavier root fresh weight as compared to non-inoculated controls as shown in Table 3-3 below. Nevertheless, the inoculation of other mycorrhizel fungi, involving *F. mosseae*, *F. caledonius* and the commercial strain *Myke*, on wheat Snowbird and wheat 13NQW1265 generally had no significant effects on fresh root weight, presenting as no significant differences on root fresh weight between inoculated and non-inoculated controls. The only exception lay in *F. mosseae* inoculated wheat Snowbird, who responded positively to *F. mosseae* and obtained a significant heavier root fresh weight than non-mycorrhizal Snowbird. However, when wheat

FL62R1 and Scotia were taken into consideration, the results unfortunately pointed out that plant mycorrhization made no significant differences on root fresh weight between inoculated and non-inoculated samples. Furthermore, it was interesting to notice that even though mycorrhizal strain *Rhizophagus irregularis* (DAOM240442) and the commercial strain *Myke* (DAOM197198) belonged to the same arbuscular mycorrhizal species, the performance of commercial strain *Myke* (DAOM197198) was apparently worse than *Rhizophagus irregularis* (DAOM240442), particularly in wheat FL62R1 and wheat Scotia. The fresh root weight of *Rhizophagus irregularis* inoculated FL62R1 was significantly higher that *Myke* inoculated wheat FL62R1. Similarly, a heavier root fresh weight was observed in Scotia wheat inoculated by *Rhizophagus irregularis* than by *Myke*. From the aspect of wheat varieties, it can also be summarized that wheat FL62R1 (inoculated and non-inoculated) generally had the highest level of fresh root weight while wheat Snowbird (either mycorrhizae inoculated or non-inoculated) had the lowest fresh root weight. It pointed out that wheat cultivar FL62R1 was the best option among these four selected spring wheat genotypes in gaining stronger root systems.

----Root dry weight

Root dry biomass was affected by variances in wheat varieties (p-value<0.0001), arbuscualr mycorrhizal strains (p-value<0.0001) and salinity treatments (p-value 0.0234), independently (Table 3.4 and Table 3.5). In the evaluation of wheat varieties, the results demonstrated that wheat FL62R1 had the heaviest root dry weight, no matter inoculated by mycorrhizal fungi or not. The Snowbird wheat had the lowest root dry biomass while wheat Scotia and wheat 13NQW1265 had a moderate dry root biomass, which was lower than wheat FL62R1 but higher than wheat Snowbird. A comparison between wheat colonized by mycorrhizal strains *F.mosseae*, *F.caledonius*, *R.irregularis* and non-mycorrhizal controls demonstrated mycorrhiza inoculation significantly

improved root dry weight. The only exception lay on the commercial AMF strain *Myke*, in which *Myke* colonization had no significant effects on wheat varieties as compared to the non-inoculated controls.

3.4.2 Root morphology

Root total length, root surface area (SA) and root total volume were measured as representatives of root morphological variables. All three root architecture parameters were affect by variances in wheat varieties and mycorrhizal strains independently as shown in Table 3.4 below, without any two-way interaction. Generally, the results indicated that inoculation of *Rhizophagus irregularis* (DAOM240442) benefited host wheat by strengthening root development, resulting in a relatively larger root total volume, longer root length and greater root surface area. Taken root surface area as an example, a comparison between *Rhizophagus irregularis* inoculated wheat samples and non-mycorrhizal controls illustrated that with a significant bigger root surface area, colonization of *Rhizophagus irregularis* fungus was believed to positively promoted root growth and development. From the aspect of wheat varieties selection, the results illuminated that wheat cultivars performed differently. But wheat FL62R1 and Scotia had the best performance on all variables, resulting in highest root length, largest root surface area and largest root volume while the commonly used Snowbird wheat had the worst response, with lowest values on all root morphology variables measured.

3.4.3 Salinity effects

Salinity was the stress condition discussed in this study. According to the results we have gotten, salinity have a non-significant effect on most measured variables, except for grain yield, root dry weight and root surface area (Table 3.2 and 3.4). In addition to negatively affecting grain yield through interacting with variance in mycorrhizal strains as already discussed above, salinity

treatments also had significantly negative effects on root surface area and root dry weight. To be specific, root surface area and root dry weight decreased significantly under the detrimental effects of highest concentration of saline treatment (100 mmol/L) by comparing with the non-salt treatment in all wheat-mycorrhizal combinations and the decrease could be well described by linear regression. The founding suggested that the even the high saline stresses applied in this study may be far below the salt sensitivity of wheat cultivars and made effects of salt non-significant on most variables.

3.5 Discussion and conclusion

3.5.1 Plant biomass and root morphology

Taking together, the above findings demonstrated that mycorrhizal strains *Funneliformis mosseae* and *Rhizophagus irregularis* (DAOM 240442) generally had the best performance in terms of both plant biomass (grain yield and root biomasses) and root morphology among four selected arbuscular muycorrhizal strains. Wheat colonized by these two fungal strains showed significant advantages not only in mitigation of saline-induced yield loss but also in the promotion of root development with heavier root biomass and optimized root architecture when comparing with the non-inoculated controls. However, the performance of mycorrhizal strains *Funneliformis caledonius* and *Myke* varied among the variables.

From the aspect of plant biomass, the results illustrated that wheat inoculated by mycorrhizal fungi *Funneliformis mosseae* and *Rhizophagus irregularis* (DAOM 240442) alleviated yield loss caused by saline stress and gained heavier root biomasses. These effects may partially due to the integrated effects, which included increased phosphorus uptake, which functioned as the crucial nutrient and material base for plant development and growth; the enhanced water absorption and use efficiency to fight against salinity-induced physiology water shortage; as well as the improved photosynthesis

efficiency that provided more carbonates and facilitated transportation rate between plant-mycorrhizal symbiosis (Colla et al., 2007; Sheng et al., 2008; Talaat and Shawky, 2013).

It has been widely studied that mycorrhizae help plants improve their tolerance against salinity stresses through multiple mechanisms, ranging from root morphologic alternation to water and nutrients uptake enhancement and the comprehensive effects finally improve plant developmental vigor and gain hosts a heavier root biomass as compared to non-mycorrhizal controls under saline condition (Evelin et al., 2009; Porcel et al., 2012). However, evaluations on root biomass and yield production are not indicative enough for a full understanding of the influence of plant mycorrhization. As previous reported by Iman et al in 2006, root morphology may get changed without significant differences in root biomass (Iman et al., 2006). Therefore, in addition to root biomass, evaluation of root morphological parameters is also inevitably important.

An optimized root architecture was observed in wheat inoculated by mycorrhizal strain *Rhizophagus irregularis* (DAOM 240442), representing as larger root surface area, longer root length and bigger root total volume. Particularly, *R. irregularis* inoculated wheat had a significant larger root surface area than non-inoculated controls, which provided bigger contact areas with soil matrix, allowing plants to absorb mineral nutrient and water more efficiency, an absolute advantage in stress condition. Similarly, roots with larger total volume and longer root length were also of great value in nutrient absorption, by exposing roots to larger area of soil matrix and by drilling deeper into soil matrix for more available nutrition (Braunberger et al., 1991; Jones et al., 1989). The enhanced nutrient uptake, especially phosphorus ion, played in crucial role in the promotion of root development and resulted in the increased root fresh and dry weight in this study. Therefore, it is reasonable to conclude that colonization of mycorrhizal strain *R. irregularis* significantly optimized root architecture, which was the structural foundation of an increased

nutrient uptake capacity and the increased water and mineral absorption contributed to a heavier root biomass. However, the performances of other AMF strains were not as stable and positive as *R. irregularis*.

A comparison between wheat varieties demonstrated that the wheat FL62R1 performed best in most cases, showing significant heavier root biomass and better optimized root architecture. While the Snowbird wheat had the worst growth status (Table 3.4). Variables including root length, root surface area, root total volume and root dry weight were taken as examples to explain the advantages of the wheat FL62R1. To be specific, a comparison between means of root length of the four wheat varieties showed that the wheat FL62R1, Scotia and 13NQW1265 had significantly longer root length than wheat Snowbird, in which the smallest means of root length was observed. In the case of root surface area and root total volume, the wheat FL62R1 had significant larger values than the wheat Snowbird and the wheat 13NQW1265. While the Snowbird wheat had the smallest means of root surface area and total root volume among the four selected spring wheat cultivar as presented in table 3.4 below. Similar result was observed in root dry weight. The wheat FL62R1 gained heaviest dry root biomass among the four wheat cultivars. The wheat Scotia and 13NQW1265 had moderate root dry biomass, which was lower than wheat FL62R1 but higher than wheat Snowbird. The lowest root dry weight, however, was observed in wheat Snowbird. Taken all together, it was reasonable to conclude that the wheat FL62R1 had the best performance, representing by better optimized root architecture and enhanced root dry biomass. The optimized root architecture, such as longer root length, bigger root surface area and larger root total volume, increased chances of nutrient uptake by expanding the contact area between root and soil and therefore contributed to heavier root biomass as explained above. These findings therefore gave light to the hypothesis that in term of better response to plant mycorrhization and stronger stress

tolerance capacity, wheat FL62R1 was the most suitable choice among the four experimental wheat varieties, while the commercially used Snowbird wheat was the last option.

3.5.2 Salinity treatment

Salinity had significant negative effects on grain yield, root surface area and root dry weight when highest salinity level (100 mmol/L) was applied, but had no significant effects on other variables. One putative explanation is that salinity levels used in this study were too low to induce sufficient stress for most wheat varieties, a moderate salinity tolerance crop. Previous researches on salinity tolerance of wheat used a wide range of salt levels, ranging from 0 mM (control) to 200 mM or even higher (Zair et al., 2003; El-Hendawy et al., 2005; Tammam et al., 2008) and generally significant negative effects would be observed when saline solution had a concentration higher than 150 mmol/L. For instance, Tamman et al. (2008) reported that salinity levels applied on a moderately salinity-tolerant wheat variety can range from 0 mmol/L to 320 mmol/L and generally significant changes would be found in most variables with salt solutions higher than 120 or 180 mmol/L. Thus it is reasonable to deduce that the 100 mmol/L of NaCl used in our study did not provide sufficient stress for wheat, leading to insignificant effects of salinity on more variables. But the finding still provided useful information. If we consider the highest salt solution (100 mmol/L, which equaled to 5.8g/L, slightly saline soil condition) we used as saturation extract from soil, it would be possible to deduce that these four wheat varieties are able to grow on slightly saline soil without negative effects (Brouwer et al., 1985).

Table 3.1 Wheat varieties, arbuscular mycorrhizal (AM) strains, salinity treatments of the experimental design		
Spring wheat varieties		
Wheat variety	Grain colour	Seeds per pot
FL62R1	Red	5
Scotia	Red	5
Snowbird	White	5
13NQW1265	Purple	5
Arbuscular mycorrhizal strains		
AM strain *	DAOM †	Number of propagules per pot
Funneliformis mosseae	198274	50
Funneliformis caledonius	242686	50
Rhizophagus irregularis	240442	50
Myke	197198	50
Salinity treatments		
Saline solution (mmol/L)	grams of NaCl (/L)	Volume of saline solution per pot (L)
0	0	0.1
50	2.925	0.1
100	5.85	0.1

* The mycorrhizal strains *Rhizophagus irregularis* (DAOM 240442) and *Myke* (DAOM 197198) originate from the same mycorrhizal species but different strains.

†DAOM, Canadian National Mycological Herbarium

Table 3.2 Effect of interaction between mycorrhizal strains and salinity levels on grain yield, sliced by mycorrhizal strains (α = 0.05)

Mycorrhizal strains	Salinity levels (mmol/L)	*p*-value	Grain yield (estimates)
Funneliformis mosseae	0	*P*=0.5024	0.6534*a*
	50		0.7682*a*
	100		0.6935*a*
Funneliformis caledonius	0	*P*=0.0027	0.8746*a*
	50		0.8243*a*
	100		0.5577*b*
Rhizophagus irregularis	0	*P*=0.3737	0.7011*a*
	50		0.8326*a*
	100		0.7961*a*
Myke	0	*P*=0.2865	0.7156*a*
	50		0.5693*a*
	100		0.6765*a*
Control	0	*P*=0.0205	0.8390*a*
	50		0.6743*ab*
	100		0.5667*b*
Mycorrhizal strains * salinity levels		*P*=0.0121	

[1]Grain yield is presented as estimates, because there was difficulty with the transfer back into means

[2] A two-way-interaction between mycorrhizal strains and salinity levels was found

[3] Estimates with the same letters are not significantly different with 95% confidence limits

Table 3.3 Effect of interaction between mycorrhizal strains and wheat varieties on root fresh weight, sliced by wheat varieties (α = 0.05)

Wheat varieties	Mycorrhizal strains	*p*-value	Root fresh weight (means)
FL62R1	*Funneliformis mosseae*	*p* =0.0425	2.4593*ab*
	Funneliformis caledonius		2.6365*ab*
	Rhizophagus irregularis		3.0955*a*
	Myke		2.1972*b*
	Control		2.6191*ab*
Scotia	*Funneliformis mosseae*	*p* =0.0179	2.1776*ab*
	Funneliformis caledonius		2.1834*ab*
	Rhizophagus irregularis		2.2746*a*
	Myke		1.7020*b*
	Control		2.1886*ab*
Snowbird	*Funneliformis mosseae*	*p* =0.0016	1.8465*a*
	Funneliformis caledonius		1.3465*ab*
	Rhizophagus irregularis		1.8073*a*
	Myke		1.6429*ab*
	Control		1.2751*b*
13NQW1265	*Funneliformis mosseae*	*p* =0.0108	2.2588*ab*
	Funneliformis caledonius		2.0449*ab*
	Rhizophagus irregularis		2.4982*a*
	Myke		1.9780*ab*
	Control		1.9036*b*
Mycorrhizal strains * wheat varieties		*p*-value=0.0137	

[1] Root fresh weight is presented as means

[2] A two-way-interaction between mycorrhizal strains and wheat varieties was found

[3] Means with the same letters are not significantly different with 95% confidence limits

Table 3.4 Back-transferred means for root morphology and root dry weight with Bonferroni grouping (α = 0.05)

	Root length (mm)	Surface area (mm*mm)	Root volume (mm*mm*mm)	Root dry weight (grams)
Wheat varieties				
FL62R1	280.07*a*	84.7314*a*	2.0491*a*	0.5401*a*
Scotia	287.98*a*	79.0213*ab*	1.725*ab*	0.4618*b*
Snowbird	239.03*b*	58.6932*c*	1.2598*c*	0.3544*c*
13NQW1265	304.79*a*	75.2293*b*	1.438*bc*	0.437*b*
p-value	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001
Mycorrhizal strains				
F.mosseae	301.60*a*	81.2535*ab*	1.744*ab*	0.4732*a*
F.caledonius	252.54*bc*	64.8801*cd*	1.3384*c*	0.4556*a*
R.irregularis	320.13*a*	88.5825*a*	1.9599*a*	0.5121*a*
Myke	237.81*c*	63.7823*d*	1.3682*bc*	0.392*b*
Control	280.57*ab*	73.19*bc*	1.696*abc*	0.3959*b*
p-value	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001

[1] *F.mosseae=Funneliformis mosseae*; *F.caledonius=Funneliformis caledonius*; *R.irregularis=Rhizophagus irregularis*

[2] The four variables were analyzed based on their different distributions and thus means here were back-transferred means

[3] No two-way-interaction are found in these four variables, they are affected by variance in wheat varieties and mycorrhizal strains independently

[4] Means with the same letters are not significantly different with 95% confidence limits

Table 3.5 Back-transferred means for root surface area and root dry weight under salinity effects with Bonferroni grouping ($\alpha = 0.05$)

Salinity levels (mmol/L)	Surface area (mm*mm)	Root dry weight (grams)
0	76.9581*a*	0.4615*a*
50	74.4342*ab*	0.4493*ab*
100	69.9915*b*	0.4203*b*
p-value	*p*=0.0200	*p*=0.0234

[1] The two variables were analyzed based on their different distributions and thus means here were back-transferred means

[2] No two-way-interaction are found and therefore they are affected by variance in salinity levels independently

[3] Means with the same letters are not significantly different with 95% confidence limits

Connecting text

In the chapter 3 above, we have discussed the responses of four selected spring wheat cultivars to the colonization of four arbuscular mycorrhizal fungi under salinity treatments. Based on the results, we concluded that AMF *Rhizophagus irregularis* (DAOM 240442) performed the best in all ways, as represented by significantly alleviating yield loss caused by saline stress and positively gaining a stronger root system. The salinity stress unexpectedly had no significant effects, neither positive nor negative, on most variables apart from grain yield, root surface area and root dry weight. Therefore, in the following modified study as stated in chapter 4, salinity treatments were removed.

This modified second experiment evaluated the responses of the same four spring wheat varieties to the colonization of two selected arbuscular mycorrhizal strains instead of four, namely *R. irregularis* (DAOM 240442), the best performing fungal strain in the previous study and the commercial product *Myke* (DAOM 197198), which contains a different strain of *Rhizophagus*. This study was designed to further support previous results. These results were submitted to the Journal of Plant Nutrition for review.

*D. Yi, T. Schwinghamer, Y. Dalpé, J. Singh and S. Khanizadeh, 2016: Response of spring wheat (*Triticum aestivum* L.) varieties to arbuscular mycorrhizal colonization. Journal of Plant Nutrition.

Chapter 4

Response of spring wheat (*Triticum aestivum* L.) varieties to arbuscular mycorrhizal colonization

4.1 Abstract

Wheat is an important cereal crop that plays crucial roles in every aspect of human health. However, wheat production nowadays is under severe challenges from both biotic and abiotic levels. Numerous approaches have been explored and carried out to alleviate yield losses, and the application of mycorrhizal association is one of the most promising ways. Arbuscular mycorrhizal (AM) fungi have long been known to form an important mutualistic association with most plants worldwide and to benefit hosts in various aspects, including nutrient uptake promotion, material exchange facilitation as well as biotic and abiotic tolerances improvement. The central objective of this study, therefore, was to understand and compare the influence of two selected AM fungi on four spring wheat varieties (*Triticum aestivum* L.). Plant biomass and root morphologic parameters were measured and analyzed using PROC GLIMMIX (Generalized Linear Mixed Models) in the SAS software package. The statistic analysis results reveal that AMF strain *Rhizophagus irregularis* (DAOM 240442) consistently had a positive effect on wheat. *Rhizophagus irregularis* inoculated wheat cultivars had a significantly higher root-to-shoot ratio, a longer root system, a larger root surface area, and heavier fresh and dry root weights as compared to non-inoculated wheat controls. The commercial strain *Myke* (DAOM 197198) performed neutrally, without significant differences from controls in most cases. The results further suggest that with respect to wheat variety comparison, the commonly used wheat Snowbird was the last choice, performing worst in the majority of variables measured.

4.2 Introduction

Wheat (*Triticum aestivum* L.), ranking after rice and maize, is among the top three most productive cereal crop worldwide (FAOSTAT 2013). Planted on more than one sixth of the farmland acreage globally, wheat provides food to approximately half of the global population (Gupta et al. 2008). In 2013, global production of wheat amounted to more than 0.71 billion tonnes, versus 0.74 billion tonnes of rice and over 1 billion tonnes of maize (FAOSTAT 2013). Wheat plays an important role in Canadian agriculture as well. For example, Canada produced over 37 million tonnes of wheat and created a net production value of more than $5 billion in 2013 (FAOSTAT 2013). Wheat appears on humans' dining tables every day in the form of bread, pasta, pizza and beer, etc. In addition, wheat also has significant medicinal value. Previous studies have reported that wheat consumption lowers chances of numerous chronic diseases, including diabetes (Meyer et al. 2000), organ cancers (Kasum et al. 2002, Jacobs et al. 1995) and cardiovascular disease (Jacobs et al. 1998). Moreover, even wheat waste is of great use, either used in livestock feeding or used as feedstock for biofuel synthesis (Tishler et al. 2015). However, wheat production is severely challenged nowadays both by abiotic stresses such as soil salinization, drought and extreme weather conditions and by biotic stresses from bacteria, fungi and virus infection. Numerous approaches have been taken to alleviate productivity losses, such as selection of tolerant varieties (Mozafar and Goodin 1986) and exploration of beneficial symbiosis (Beltrano and Ronco 2008). The application of mycorrhizal fungi symbiosis is one of the most promising approaches that attracts increasing public attention.

Mycorrhizae are mutualistic associations that form between fungi and most terrestrial plants in their roots (Kirk et al. 2001). Two major types of mycorrhizae have been found, namely endomycorrhizae such as arbuscular mycorrhizae (AM), in which fungal hyphae puncture host

root cells; ectomycorrhizae, in which fungal hyphae remain between root cells of host plants instead of puncturing into root cells. It has been widely reported in previous studies that AM associations benefit plants mainly by increasing water and mineral nutrients absorption, especially P, N, K, Zn and Cu (by 80%, 25%, 10%, 25% and 60%, respectively) (Marschner and Dell 1994). Moreover, there are evidences from earlier studies that AMF inoculation increases host biotic and abiotic tolerances (Al-Karaki et al. 2004; Daei et al. 2009; Pozo et al. 2010).

Therefore, the main target of this study was to identify and to compare the influence of two arbuscular mycorrhizal fungi on four selected spring wheat varieties.

4.3 Material and Methods

A previous study conducted by the present research team found that AMF strain *Rhizophagus irregularis* (DAOM 240442) had the best performance among four selected mycorrhizal strains, as supported by its strongest capacity in mitigation of saline-induced yield losses and facilitation of root development to gain stronger root systems. Furthermore, with respect to wheat variety screening, the results suggest that wheat variety FL62R1 performed better than other varieties by gaining a stronger root system. Finally, salinity unexpectedly had no significant effects on most root morphologic variables, which was possibly because the salinity stresses that we used did not provide sufficient stresses for those studied spring wheat.

Therefore, based on the findings of that previous study, a modified second experiment was designed for the present study. This new experiment evaluated the responses of the same four spring wheat varieties, namely, FL62R1, Scotia, Snowbird and 13NQW1265, to the colonization of two selected mycorrhizal strains instead of four (Table 4.1), namely, *R. irregularis* (DAOM 240442), the best performed fungus strain in our previous study and the commercial product *Myke* (DAOM 197198), which originates from the same fungus species with *R. irregularis* but a

different strain. Arbuscular mycorrhizal strains were provided by Dr. Yolande Dalpé from Ottawa Research and Development Centre, Agriculture and Agri-Food Canada.

The study was conducted in a regulated greenhouse with a 16-h photoperiod at a day/night temperature regime of 22 °C/20 °C. For each wheat variety, five seeds inoculated with 50 spores of mycorrhizal fungi were planted per 6-inch pot with equal distance between seeds, along with a non-inoculated controls. Inoculants were provided in form of specific-spore-containing matrix with the concentration of spores detected. Non-mycorrhizal controls were treated with mycorrhizal-free AGRO MIX G10 soil instead of mycorrhizal-spore-containing matrix. AGRO MIX G10 soil (AF) was used, which ensures no extra mycorrhizal fungi in soil. With five replications per treatment, a total of 60 pots were set up in a regulated greenhouse following the randomized complete block design (RCBD). Constant irrigation was provided throughout whole growth period and plants were fertilized (a fertilizer named 20-20-20) every two weeks with 100 ml per pot with a concentration of 1% (1 gram of fertilizer 20-20-20 into 1 liter of water).

Plants were harvested and variables were measured ten weeks after planting when plants got fully matured. Data on plant biomass and root morphology (grain yield, root fresh, root dry weights, root length, root average diameter, root surface area and root total volume) were collected and analyzed using PROC GLIMMIX (Generalized Linear Mixed Models) of the SAS software package.

4.4 Results

4.4.1 Plant biomass

Grain yield and root-to-shoot ratio were measured and analyzed with respect to plant biomass. The PROC GLIMMIX output demonstrates that grain yield was significantly affected by wheat

varieties with a *p*-value of 0.0023 (Table 4.2). Specifically, the wheat variety Scotia had the highest yield (2.6954 g), whereas the purple line 13NQW1265 had significantly lower yield (1.7896 g) as compared to wheat Scotia. The wheat varieties Snowbird (1.9704 g) and FL62R1 (2.0231 g) had moderate performance in terms of grain production, without significant differences from either Scotia or 13NQW1265.

The root-to-shoot ratio analysis results illustrate that interaction between AMF strains and wheat varieties significantly affected plant root-to-shoot ratio with a *p*-value of 0.0152 (Table 4.3). Root-to-shoot ratios of wheat FL62R1 ($p = 0.0149$) and wheat 13NQW1265 ($p = 0.004$) responded dramatically but inconsistently to mycorrhizal colonization. Specifically, *R. irregularis* inoculation positively facilitated root growth and development of wheat cultivar FL62R1, resulting in a significantly higher root-to-shoot ratio in comparison with that of the non-inoculated controls. But a comparison between *R. irregularis*-inoculated wheat 13NQW1265 and controls illustrated that wheat 13NQW1265 reacted non-significantly to mycorrhizal strain *R. irregularis*. In contrast, wheat 13NQW1265 responded negatively to *Myke* inoculation, resulting in a prominently lower root-to-shoot ratio than non-inoculated controls. But colonization of the commercial AMF strain *Myke* had no significant effects on wheat FL62R1. Nevertheless, neither *R. irregularis* nor *Myke* had any significant effects on root-to-shoot ratios of wheat Scotia and wheat Snowbird. With respect to comparison of wheat varieties, the results suggest that a significant difference on root-to-shoot ratio between wheat varieties was only observed with the disappearance of mycorrhizas, in which non-inoculated wheat 13NQW1265 had a significantly higher root-to-shoot ratio than other non-inoculated cultivars. The formulation of mycorrhizal associations evanished the differences. Therefore, it could be deduced that colonization of AMF strains *R. irregularis* and *Myke* helped eliminate discrepancy in root-to-shoot ratio caused by wheat varieties difference.

4.4.2 Root morphology

Furthermore, the results show that mycorrhizal symbiosis, especially the AMF strain *R. irregularis*, significantly promoted root morphological optimizations in comparison with the non-inoculated controls, resulting in longer root length, greater root surface area, smaller root average diameter, and heavier fresh and dry root weights. Taking root surface area as an example, the results indicate that root surface area was significantly influenced by the interaction between wheat varieties and mycorrhizal strains with a *p*-value of 0.0039 (Table 4.3). Inoculation of mycorrhizal strain *R. irregularis* significantly enhanced root surface area in wheat varieties FL62R1, Scotia and Snowbird but not in wheat 13NQW1265 as compared to the non-inoculated controls. In contrast, the commercial strain *Myke* had non-significant effects on root surface area of most varieties except for wheat Snowbird. Specifically, *Myke*-inoculated Snowbird wheat gained significantly higher root surface area than controls. But there was no significant increasing in root surface area of *Myke*-inoculated wheat FL62R1, wheat Scotia and wheat 13NQW1265 in comparison with controls. With respect to wheat variety screening, the results demonstrated that in terms of higher root surface area, wheat Scotia performed best while wheat Snowbird performed worst. It could be further concluded that the commercial mycorrhizal product *Myke* was able to benefit host wheat by eliminating variations in root surface area caused by differences between wheat varieties. Specifically, wheat Scotia had a significantly higher root surface area than wheat Snowbird when inoculated by *R. irregularis* and in non-inoculated controls. However, when commercial strain *Myke* was applied, differences between wheat varieties were eliminated (*p*-value of 0.0664), resulting in similar levels of root surface area in all four wheat varieties.

Root average diameter was also of great importance in the evaluation of function of mycorrhizal symbiosis. Generally, the stronger the root systems are, the smaller the root average diameter

should be, which is partly due to the fibrous root system of wheat. Based on the statistic analysis results, wheat root average diameter was affected independently by variance in wheat varieties ($p = 0.0213$) and mycorrhizal strains ($p = 0.0003$) (Table 4.2). The FL62R1 wheat gained a significantly larger root average diameter than wheat 13NQW1265, regardless of inoculated or non-inoculated by AMF strains. The wheat varieties Snowbird and Scotia had moderate root average diameters, without significant differences from either the champion wheat FL62R1 or wheat 13NQW1265, which had the smallest root average diameter. The finding above therefore indicated that wheat 13NQW tended to have a stronger root system from the aspect of root area diameter. With respect to comparison between mycorrhizal strains, the data clearly confirmed that colonization of mycorrhizal strain *R. irregularis* positively benefited wheat root development, representing by a significantly smaller root average diameter than controls and *Myke*-inoculated wheat. Inoculation of mycorrhizal fungus *Myke*, without significant differences from non-inoculated control wheat, had no obvious influence on host root average diameter. Similar observations were found in root length: AMF strain *R. irregularis* significantly promoted hosts' root growth and development, with longer roots than the non-inoculated controls.

In addition to the root morphology variables mentioned above, root fresh and dry weights were two more plant biomass indexes that is of great value in the evaluation of AM symbiosis. Similar to root average diameter, root fresh and dry weights were significantly affected by variance in wheat varieties and mycorrhizal strains independently (Table 4.2). The results demonstrate that the wheat varieties 13NQW1265 and Scotia had significantly heavier fresh and dry weights than wheat Snowbird, which had the lowest fresh and dry root weights among the four selected spring wheat cultivars. With respect to the screening of AMF strains, *R. irregularis* inoculation positively facilitated root systems, contributing to significantly heavier fresh and dry weights in inoculated

wheat plants than in non-inoculated controls. A comparison between *Myke*-inoculated wheat and controls illuminated that the commercial AMF strain *Myke* had non-significant effects on fresh and dry root weights of host wheat varieties.

4.5 Discussion and Conclusion

The results presented above revealed the facts that formulation of arbuscular mycorrhizal symbiosis, especially with *R. irregularis* fungus (DAOM 240442), promoted plant growth and development as a consequence of comprehensive effects from root biomass facilitation and root morphologic optimization. The *R. irregularis*-inoculated wheat tended to have heavier fresh and dry root weights heavier root fresh and dry weights as well as a greater root-to-shoot ratio in comparison to non-mycorrhizal wheat controls. These advantageous alternations may due to the increased absorption of phosphorous, one of the most important mineral nutrients required by plans, by mycorrhizal fungi colonization. Arbuscular mycorrhizas have long been reported in previous researches to benefit hosts by boosting the translocation and transferring of soil nutrition close to root cells and the efficient absorption (Bolan, 1991; Vance 2003). The increased absorption and utilise of P element in plants then attribute to bigger root system with heavier root biomass.

However, root biomass is not indicative enough to explain the changes in root architecture. Iman et al. (2006) has reported in his research that alternation in root architecture can happen without changes in root biomass. Then how mycorrhizal colonization alters root morphology and how these alternations enhance phosphorous absorption in root cells? To answer these questions, root morphologic parameters were also measured in this study. The results demonstrate that colonization with *R. irregularis* fungus contributed to stronger root systems, as evidenced by longer roots, larger root surface area and smaller root average diameter in *R. irregularis*-wheat than in non-mycorrhizal controls. Roots length, surface area and average diameter are three

determinative metrics for root architecture and plant growth and is significant associated to P absorption and usage. Usually, roots with smaller average diameter result from high proportion of root hairs, which help roots get into small pores in soil easier and therefore increase soil volume roots are exposed to and finally get chances to absorb more phosphate ions from soil solution. Similarly, roots with bigger surface area provide higher contact area for phosphate ions absorption. It gets supported by former studies that the longer and thinner the roots are, the more efficiency phosphorous could be obtained from soil matrix (Braunberger et al. 1991; Jones et al. 1989). Therefore, it is reasonable to make a conclusion that colonization of mycorrhizal strain *R. irregularis* benefited wheat mainly through improvement of phosphorous absorption as a consequence of optimized root architecture and thus contributes heavier root biomass. However, the commercial AM strain *Myke* performed neutrally in most variables, without significant differences from non-mycorrhizal wheat controls. The conclusions were consistent with our preliminary results showing that wheat benefited most from inoculation of *R. irregularis*, representing as heavier plant biomasses and more favorable root morphology. The performance of the commercial strain *Myke*, however, varied among traits.

With respect to wheat varieties evaluation, the results demonstrate that the commonly used wheat cultivar Snowbird responded worst in case of most variables, resulting in lower root fresh and dry weights, a smaller root-to-shoot ratio (especially without mycorrhizal inoculation), shorter root length and smaller root surface area. It was consistent with our preliminary results, suggesting that Snowbird wheat may not be an optimum species resources when higher plant biomass and stronger root morphology are considered. However, no excellent wheat variety was selected from these four studies wheat genotypes, given that the performance of the wheat varieties FL62R1, Scotia and 13NQW1265 varied dramatically among traits.

Conclusively, the results of the current study indicate that the arbuscular mycorrhizal strain *R. irregularis* is a better candidate than the commercial strain *Myke* in peruse of stronger root systems. It inspires scientists that one possible next step is the commercialization of *R. irregularis* as a substitution for AMF strain *Myke*. Moreover, further molecular work need be designed to understand the molecular background for the interaction between plants and mycorrhizal fungi and for the identification of genes responsible for mutualism.

Table 4.1 Wheat varieties, arbuscular mycorrhizal fungi (AM) strains, and number of seeds and spores added to each pot

Four selected spring wheat varieties		
Wheat variety		Seeds per pot
FL62R1		5
Scotia		5
Snowbird		5
13NQW1265		5
Two screened arbuscular mycorrhizal fungi strains		
AM strain[1]	DAOM[2]	Number of spores per pot
Rhizophagus irregularis	240442	50
Myke	197198	50

[1]The mycorrhizal strains *Rhizophagus irregularis* (DAOM 240442) and *Myke* (DAOM 197198) originate from the same mycorrhizal species but different strains.

[2]DAOM, Canadian National Mycological Herbarium.

Table 4.2 Means for grain yield, root fresh and dry weights, and root average diameter with Bonferroni grouping ($\alpha = 0.05$)

	Grain yield[1] (g)	Root fresh weight (g)	Root dry weight[2] (estimated)	Root average diameter (estimated)
Wheat varieties				
	$p = 0.0023$[3]	$p < 0.0001$	$p = 0.0009$	$p = 0.0213$
FL62R1	2.0231 ab[4]	1.2248 ab	0.3649 ab	0.09845 a
Scotia	2.6954 a	1.4896 a	0.4215 a	−0.07244 ab
Snowbird	1.9704 ab	0.9832 b	0.3254 b	−0.01259 ab
13NQW1265	1.7896 b	1.5036 a	0.4001 a	−0.01660 b
Mycorrhizal strains[5]				
	$p = 0.6019$	$p = 0.0118$	$p = 0.0160$	$p = 0.0003$
***R. irregularis*[5]**	1.9495 a	1.4566 a	0.4117 a	−0.2014 b
Myke	2.1194 a	1.2701 ab	0.3724 ab	−0.00704 a
Control	2.0939 a	1.1377 b	0.3500 b	0.1129 a

[1]Grain yield and root fresh weight are presented as means

[2]Root dry weight and root average diameter are presented as estimates, because there was difficulty with the transfer back into means

[3]No interaction was found in these four variables. They were affected by either wheat variety or mycorrhizal strain independently

[4]Means with the same letters are not significantly different with 95% confidence limits.

[5]*R. irregularis* = *Rhizophagus irregularis*; *Myke* = commercial strain of *R. irregularis*; control = no inoculation

Table 4.3 Effect of interaction between wheat variety and mycorrhizal strain on root-to-shoot ratio and root surface area, sliced by wheat variety ($\alpha = 0.05$)

Wheat variety	Mycorrhizal strain[1]	Root-to-shoot ratio[2] (estimates)		Root surface area[3] (mm*mm or mm^2)	
FL62R1	*R. irregularis*	$p = 0.0149$[4]	0.1461 a[5]	$p < 0.0001$	32.5262 a
	Myke		0.1029 ab		17.3689 b
	Control		0.09523 b		13.9106 b
Scotia	*R. irregularis*	$p = 0.4152$	0.1116 a	$p = 0.0090$	41.1687 a
	Myke		0.1019 a		26.9182 ab
	Control		0.08819 a		20.2483 b
Snowbird	*R. irregularis*	$p = 0.5584$	0.09580 a	$p = 0.0056$	20.2615 a
	Myke		0.07826 a		21.6457 a
	Control		0.09616 a		12.8078 b
13NQW1265	*R. irregularis*	$p = 0.0040$	0.1230 ab	$p = 0.4436$	23.4200 a
	Myke		0.1003 b		23.1656 a
	Control		0.1626 a		19.7796 a
Wheat variety × mycorrhizal strain		$p = 0.0152$		$p = 0.0039$	

[1]*R. irregularis* = *Rhizophagus irregularis*; *Myke* = commercial strain of *R. irregularis*; control = no inoculation.

[2]Root-to-shoot ratio is presented as estimates, because there was difficulty with the transfer back into means.

[3]Root surface area is presented as means.

[4]Interaction between wheat variety and mycorrhizal strain was found for both root-to-shoot ratio and root surface area.

[5]Means with the same letters are not significantly different with 95% confidence limits.

Connecting text

In the previous chapters 3 and chapter 4, we have discussed two greenhouse experiments that evaluated the response of selected spring wheat varieties to arbuscular mycorrhizal colonization under salinity stresses and under normal conditions from plant biomass and root architecture aspects. In the following chapter 5, we focused on the antioxidant capacity alternation of wheat varieties as a consequence of colonization by diverse arbuscular mycorrhizal strains. As concluded in the literature review above, mycorrhizae help plants alleviate salinity stress through multiple approaches and one most important mechanism is the enhancement of antioxidant capacity. The improved scavenging efficiency of ROS protects plants from oxidant damage, a by-product of saline stress. But there is still an enigma on the relationship between mycorrhization and enhancement of internal antioxidant capacity. Therefore, it is of great value to understand how antioxidant capacity responses to plant mycorrhization.

In the next chapter, we successfully identified the responses of total phenolic content (TPC) and DPPH scavenging capacity to variances in wheat varieties and arbuscular mycorrhizal strains. The study provides valuable information for the selection of wheat varieties with high antioxidant capacity and wheat varieties that response most positively to mycorrhization. The finding has been accepted and published in the Journal of Food, Agriculture and Environment (JAFE).

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Chapter 5

Comparison of total phenolic content and antioxidant capacity of mycorrhizal-colonized white, red and purple spring wheat (*Triticum aestivum* L.) genotypes

5.1 Abstract

Wheat (*Triticum aestivum* L.) is one of the world's most valuable crops, not only as a diet component but also as a source of dietary antioxidants. It is well known that wheat inoculated with mycorrhizal strains usually has a higher yield and stronger tolerance to stresses. However, information on the effect of mycorrhizal inoculation on the antioxidant capacity of wheat grains is scarce. The objective of this study was therefore to investigate and compare the total phenolic content (TPC) and DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging capacity of grains from four selected spring wheat varieties under colonization by four different mycorrhizal strains. Data were analyzed using PROC GLIMMIX (Generalized Linear Mixed Models) of the SAS software package. The results demonstrated that the coloured wheat, 13NQW1265, had the strongest DPPH scavenging capacity and the highest TPC in grains among all selected wheat genotypes, whereas the wheat variety Snowbird had a lower antioxidant capacity. A comparison between wheat colonized by mycorrhizal strains and non-inoculated wheat controls illustrated that the application of mycorrhizae had no significant effects on TPC in most wheat varieties; the exceptions were FL62R1 wheat inoculated with the commercial product *Myke* (*Rhizophagus irregularis*), which unexpectedly showed a negative effect, and 13NQW1265 wheat inoculated with *Funneliformis mosseae*, which increased TPC. As expected, a significant positive correlation was found between DPPH scavenging capacity and TPC, the main contributor to antioxidant capacity. The results suggest that mycorrhizal colonization could increase antioxidant compounds in wheat subject to wheat variety and mycorrhizal strain.

5.2 Introduction

The field of free radical chemistry and antioxidants has been attracting increasing attention recently. Most free radicals are chemically reactive atoms or molecules that are derived either from endogenous physiological and biochemical reactions, such as immune reactions, pathological changes to tissues, and aging, or from exogenous stimuli, including environmental pollution, drug intake, and improper cooking (Lobo et al., 2010; Pham-Huy et al., 2008). Redundancy of free radicals leads to oxidative stress and causes damage to important biological molecules, including proteins, nucleotides, and lipids (McCord 2000). This damage results in pathological changes and chronic diseases (Lobo et al., 2010; Pham-Huy et al., 2008; Rao et al., 2006). Antioxidants, however, are molecules that can stabilize free radicals by providing electrons to neutralize the unpaired electrons of free radicals and thus decrease the risks of free-radical-induced cell damage. There are four levels of antioxidant defense actions (Niki 1993). First, there are antioxidants which could prevent the generation of free radicals from the very beginning. Once free radicals have already formed, the second defense system is activated in order to inactivate the free radicals and prevent further free-radical damage by either inhibiting initiation or breaking the elongation of reaction chains. Vitamin E is one of the most well-known endogenous radical-scavenging antioxidants. If free radicals have already caused structural damage to functional molecules such as proteins and nucleotides, then the third level of defense comes into play—the removal of oxidized molecules in a timely manner to prevent the over-accumulation of detrimental free radicals in living cells. The final level of defense is the generation and transport of antioxidants to functional sites under signal stimulation from the formation and reaction of free radicals (Lobo et al., 2010; Niki 1993). Some antioxidants are formed endogenously as the products of normal metabolic processes, as reported for ubiquinol and alpha-tocopheryl hydroquinone by Shi *et al.*

(Shi et al., 1999). However, a large number of antioxidants have to be taken in from exogenous sources, either because those antioxidants cannot be formed in the body, such as vitamin C, or because the amount generated in the body cannot meet the demand (Levine et al., 1999). Synthetic and natural antioxidants are the two major groups of antioxidants that have been studied and applied in the fields of foods and medicine during the past several decades (Lobo et al., 2010; Papas 1999). However, increasing evidences have shown that synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are highly toxic to the body (Papas 1993), and thus researchers have shifted their attention to natural antioxidants that are believed to be safer for the body.

Wheat (*Triticum aestivum* L.) is one of the world's most important crops in terms of both agricultural and economic value. Wheat is the third most-produced cereal crop in the world (FAOSTAT 2013), ranking after rice and maize. Planted on over one sixth of global arable land, wheat successfully meets 40% of the food demand worldwide (Gupta et al., 2008). Previous studies have pointed out that besides being an important staple food for humans, whole wheat grains also have significant nutritive and medicinal value. The consumption of wheat helps reduce the risks of various chronic diseases such as organ cancers (Ferguson and Harris, 1999; Jeong et al., 2007; Kasum et al., 2002; Reddy et al., 2000), cardiovascular diseases (Tompson 1994; Jacobs et al., 1998; Kristensen et al., 2012), and diabetes (Anderson, 1985; Meyer et al., 2000). These health benefits are partly attributable to the presence of multiple antioxidant components in wheat, especially in wheat bran, and can be further used as an index in food quality improvement (Truswell 2002). As reported in previous studies, wheat is a good raw source of phenolic acids and thus has high levels of antioxidant capacity (Baublis et al., 2000; Onyeneho and Hettiarachchy 1992). It has been well known that the mycorrhizal colonization of wheat could improve its

tolerance to various stresses such as high salinity and drought (El-Amri et al., 2013; Ellis et al., 1985). But limited information is available on the relationship between mycorrhizal inoculation and changes in the tolerance to oxidant stresses and in the antioxidant capacity of wheat. Therefore, the aim of the present study was to investigate the total phenolic content (TPC) and the DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity of selected spring wheat varieties inoculated with special arbuscular mycorrhizal strains.

5.3 Materials and Methods

5.3.1 Experimental design

The experiment was conducted in a greenhouse under a 16-h photoperiod and a day/night temperature regime of 22°C/20°C. Four spring wheat varieties, namely, FL62R1, Scotia, Snowbird, and 13NQW1265, were used with four mycorrhizal strains, namely *Funneliformis mosseae, F. caledonius, Rhizophagus irregularis*, and the commercial product *Myke* (*Rhizophagus irregularis*) (Table 5.1), under three salinity treatments with six replications. Five seeds from each wheat cultivar were planted in 6-inch pots. Arbuscular mycorrhizal fungi were inoculated at a rate of 50 spores per pot, and other pots were left as non-inoculated controls. Thus there were 60 pots in each replication, with six replications in total. The pots were arranged using a randomized complete block design (RCBD). Beginning four weeks after planting, the seedlings were irrigated with 100 mL of NaCl solution (0, 50, or 100 mmol L^{-1}) at 3-d intervals for six weeks. The plants were harvested 10 weeks after planting.

Our preliminary analysis results for plant biomass and root morphology showed that salinity treatments had a non-significant effect on most variables. Thus, seeds from samples without salinity treatment (0 mmol/L) were collected, but the number of replications was reduced from six to three by combining each pair of replications. Seeds were kept in an oven at 60°C for 3 d to dry

them out, and wheat bran was removed before phenolic components were extracted and DPPH radical-scavenging capacity was measured. Data on TPC and DPPH scavenging capacity after four different reaction times (0.3, 10, 20, and 30 min) were analyzed using PROC GLIMMIX (Generalized Linear Mixed Models) of the SAS software package.

5.3.2 Analysis of total phenolic content and DPPH scavenging capacity

----Total phenolic content Analysis

A 0.5-g sample of whole-grain flour was extracted twice with 5 mL of 80% methanol using an IKA shaker. The extraction was carried out under nitrogen for 30 min. The tube content was centrifuged at 10,000 g for 20 min. The extracts were then pooled together into a caped culture tube, purged with nitrogen and kept frozen at −20°C until further analysis was performed.

The TPC in the wheat extracts was measured based on the Folin–Ciocalteu method described by Abdel-Aal *et al.* (2012), with ferulic acid, the main phenolic compound in wheat, used as a standard for calibration and quantification (Abdel-Aal et al., 2012). The reaction mixture contained 250 µL of sample extract, 250 µL of diluted Folin–Ciocalteu reagent, and 500 µL of saturated sodium carbonate solution. The mixture was brought up to a final volume of 5 mL with distilled water. The contents were mixed, kept in darkness for 30 min, and then centrifuged at 10,000 × *g* for 10 min before absorbance was read at 725 nm. A series of ferulic acid standard solutions were prepared at 0-16 µg/mL and read against a reagent blank. The concentrations showed a linear relationship against absorbance having a determination coefficient (R^2) 0.9869 and the results of TPC were expressed as µg ferulic acid equivalents per g sample.

----DPPH scavenging capacity

The assay for DPPH radical scavenging capacity was carried out using the stable 2,2-diphenyl-1-

picrylhydrazyl radical (DPPH•) as previously described (Abdel-Aal et al., 2012). The antioxidant reaction was initiated by transferring 1 mL of wheat extract into a test tube containing 4 mL of 80% methanol and 1 mL of freshly prepared 1 mmol DPPH• solution. The reaction mixture was monitored by reading absorbance at 517 nm for 30 min. A blank reagent was used to study the stability of DPPH• over the test time. The DPPH scavenging capacity of the wheat extracts was measured and calculated as micromoles of Trolox equivalents per 100-g sample (μMol TE $100\ g^{-1}$).

5.4 Results

5.4.1 DPPH scavenging capacity

The PROC GLIMMIX output demonstrated that the DPPH scavenging capacity of wheat grains was significantly affected by the interaction between reaction time and wheat variety ($p < 0.0001$). This indicates that for analysis of the effect of wheat varieties, different reaction times have to be considered separately. Similarly, for analysis of the effect of increasing the reaction time, the data have to be separated by wheat varieties (Table 5.2; Figure 5.1). With respect to wheat varieties, the data in Table 5.2-1 illustrate similar trends for all four varieties, showing that when the reaction time was longer, an increasing amount of DPPH free radicals could be scavenged. For wheat varieties FL62R1 and Snowbird in particular, the amount of DPPH free radicals scavenged kept increasing as the time increased. However, the situation was slightly different for Scotia and 13NQW1265 (a purple grain line): in those two wheat varieties, the DPPH radical-scavenging capacity significantly increased from 0.3 to 10 min. But after 10 min of reaction time, the value stopped increasing, and there was no significant difference in DPPH scavenging capacity between 10, 20, and 30 min. The specific reason behind the variety differences is still unknown. But, the profile of phenolic compounds in white, red and purple wheat varieties is different which could

affect the behaviour of DPPH scavenging reaction. With respect to time, as shown in Table 5.2-2, the data clearly demonstrate that no matter how long the reaction lasted, from 0.3 to 30 min, the coloured wheat variety 13NQW1265 (a purple grain line) consistently had the highest level of DPPH scavenging ability. The wheat variety Scotia had a moderate level of antioxidant capacity, whereas the wheat varieties FL62R1 and Snowbird had the lowest levels of antioxidant capacity.

5.4.2 Total phenolic content analysis

The TPC (total phenolic content) was another important index to evaluate the antioxidant capacity of an organism. The results indicate that TPC was significantly affected by the interaction between wheat variety and mycorrhizal strain (p=0.0338). The performance of the mycorrhizal strains varied considerably among the different wheat varieties (Table 4.3-1). The data clearly show that varying the mycorrhizal strains had significant effects on TPC of wheat varieties FL62R1, Snowbird, and 13NQW1265 but had no effects on Scotia. In particular, Snowbird plants whose root systems were inoculated with *F. caledonius* had significantly higher TPC than plants whose root systems were inoculated with *Myke*, the commercial mycorrhizal strain. However, a comparison between mycorrhizal inoculation and non-mycorrhizal controls showed something unexpected: there were generally no significant differences in TPC, indicating that mycorrhizal colonization neither increased nor decreased TPC as compared to non-mycorrhizal controls. However, the wheat variety FL62R1 was different. A comparison between *Myke*-inoculated wheat and non-inoculated controls revealed that *Myke* inoculation lowered grain TPC, whereas wheat inoculated with *F. mosseae*, *F. caledonius*, and *R. irregularis* performed neutrally, containing a similar level of TPC as the non-mycorrhizal controls did. The purple wheat variety 13NQW1265 performed the best: the non-mycorrhizal controls had the lowest grain TPC, whereas the wheat inoculated with *F. mosseae* had the highest TPC, significantly higher than the level in the controls.

These results clearly revealed that *F. mosseae* inoculation could increase grain TPC in the wheat variety 13NQW1265. For the wheat variety Scotia, as mentioned above, the statistical data showed that mycorrhizal colonization (with any of the strains) had no significant effects on grain TPC, hinting that Scotia responded insensitively to mycorrhizal colonization. Generally speaking, a comparison between wheat colonized by mycorrhizal strains and non-inoculated controls illustrated that the application of mycorrhizae had no significant effects on TPC for most wheat varieties, with the exception of FL62R1 with *Myke,* which unexpectedly showed negative effects, and 13NQW1265 inoculated with *F. mosseae*, which increased TPC. Furthermore, separating the interaction from the aspect of mycorrhizal strains, the data demonstrate consistently that wheat line 13NQW1265 had the highest level of TPC and that Snowbird had the lowest TPC, significantly lower than the level in 13NQW1265, whether inoculated or not with mycorrhizal strains (Table 5.3-2). Those results are consistent with those obtained for DPPH scavenging capacity.

5.4.3 Correlation analysis between TPC and DPPH scavenging capacity

Moreover, correlation analysis was done to understand the linear correlation between DPPH and TPC. There was a significant positive correlation, with a *p*-value of 0.0012, between DPPH scavenging capacity and TPC. Even though DPPH scavenging capacity and TPC were two different aspects of an organism's antioxidant capacity and might not always have exactly the same results, the correlation analysis results confirmed that in this study, grain DPPH scavenging capacity and TPC had a strong positive correlation.

5.5 Discussion and conclusion

Taken together, the results above illustrated that the DPPH radical scavenging capacity of wheat varieties increased as the reaction time increased and that this upward trend was almost not altered

by varying the wheat variety. The only exceptions were the wheat varieties Scotia and 13NQW1265, which are worth further investigation. The DPPH values for Scotia and 13NQW1265 rose significantly from 0.3 to 10 min, but after 10 min, there was no significant upward tendency. The reasons behind this observation are still unknown. One possible explanation is the inherent differences between wheat varieties. Previous studies showed that the antioxidant properties and phenolic components of wheat fractions were sensitive to and dramatically affected by wheat genotypes and environmental variations (Moore et al., 2006; Mpofu et al., 2006). Moreover, the coloured wheat variety, 13NQW1265, had the best performance in terms of antioxidant capacity, with the highest value in the DPPH assay and the highest TPC in grains. However, the commonly used wheat variety Snowbird had the lowest level of antioxidant capacity, with the worst DPPH radical quenching ability and the lowest amount of TPC. This finding was consistent with results reported in 2010 by Liu *et al.*, who found that purple wheat tended to have stronger antioxidant properties and who suggested that one possible reason for this superiority might be the significantly higher level of anthocyanins in purple wheat grains (Liu et al., 2010). With respect to TPC, despite mycorrhizal colonization (all four tested mycorrhizal strains) generally had no significant effects on the TPC in grains of those four selected spring wheat varieties, the result did figure out two sensitive combinations. Specifically, *Myke* inoculated FL62R1 wheat performed negatively, with lower TPC than non-inoculated control and 13NQW1265 wheat inoculated with *F. mosseae*, who had significantly higher TPC as compared to control. In terms of wheat varieties, it could be easily concluded that coloured wheat 13NQW1265 had excellent performance, with the highest TPC both when inoculated and non-inoculated with mycorrhizal strains, whereas Snowbird always had the worst performance. This

conclusion is not only consistent with the findings for DPPH but is also supported by a previous report (Moore et al., 2006).

Conclusively, the results of this study support the hypothesis that purple-grain wheat varieties, represented in this study by 13NQW1265, have stronger antioxidant properties than white and red-grain wheat varieties do, as evidenced by enhanced DPPH scavenging capacity and higher TPC. The commonly used variety Snowbird loses its advantage when higher antioxidant capacity is considered. Furthermore, mycorrhizal colonization, especially by strain *F. mosseae*, positively increased grain TPC in certain wheat varieties, particularly 13NQW1265, the purple grain line. However, FL62R1 inoculated with *Myke* might not be a good source of antioxidant components. These findings would be valuable new indexes not only for future wheat-breeding efforts but also in the improvement of health-beneficial components in functional foods made from wheat. One possible next step for this study would be to increase the number of samples measured in order to further explore the potential effects of mycorrhizae on antioxidant properties.

Table 5.1 Wheat varieties, arbuscular mycorrhizal (AM) strains, and amount of seeds or propagules added to each pot (with 95% confidence limits)		
Four selected spring wheat varieties		
Wheat variety	Grain colour	Seeds per pot
FL62R1	Red	5
Scotia	Red	5
Snowbird	White	5
13NQW1265	Purple	5
Four screened mycorrhizal strains		
AM strain *	DAOM †	Number of propagules per pot
Funneliformis mosseae	198274	50
Funneliformis caledonius	242686	50
Rhizophagus irregularis	240442	50
Myke	197198	50

* The mycorrhizal strains *Rhizophagus irregularis* (DAOM 240442) and *Myke* (DAOM 197198) originate from the same mycorrhizal species but different strains.

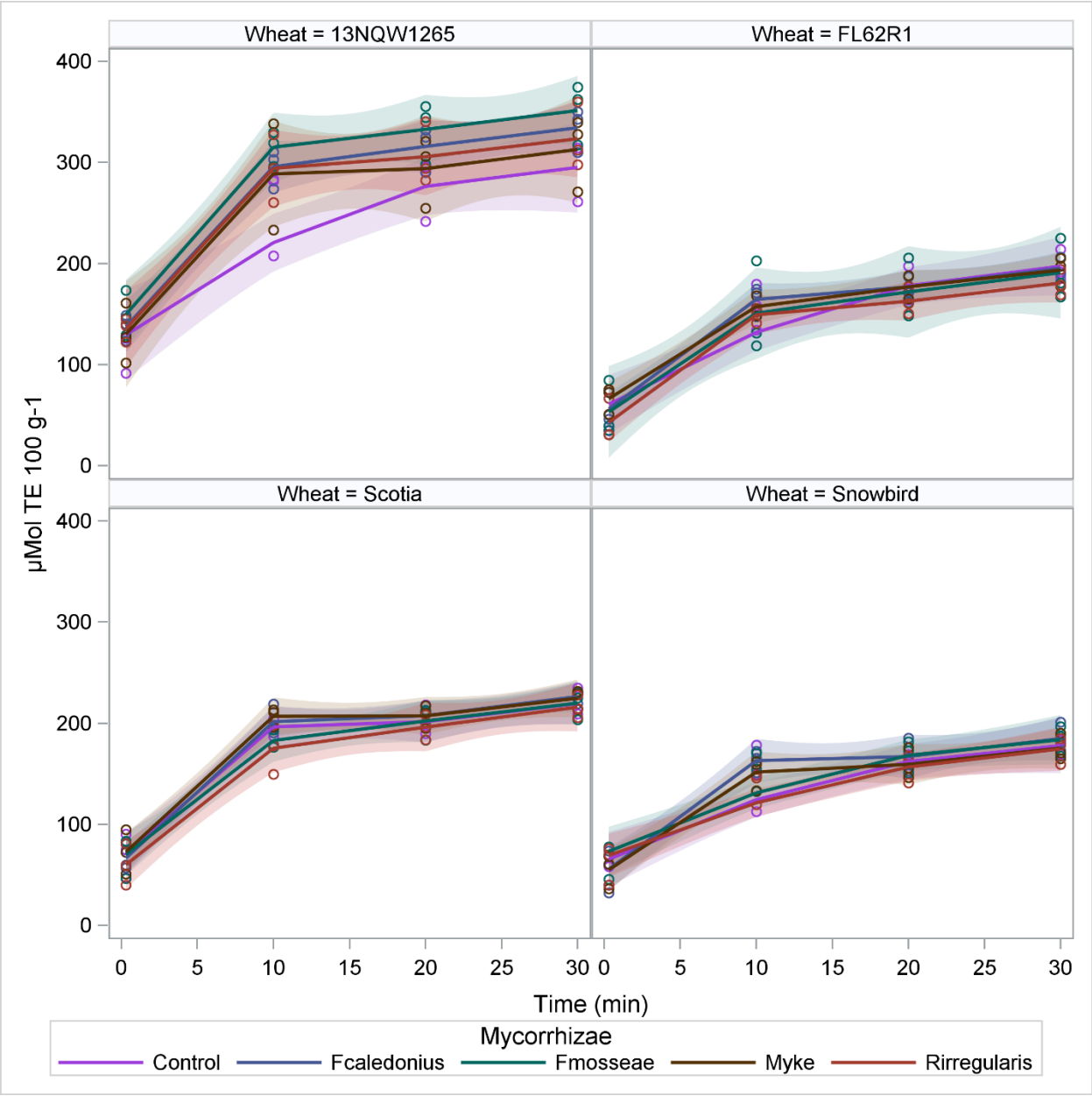
†DAOM, Canadian National Mycological Herbarium

Table 5.2 Effect of interaction between wheat variety and reaction time on DPPH radical scavenging capacity, sliced by wheat variety (with 95% confidence limits)		
Wheat variety	Reaction time (min)	Estimate for DPPH radical scavenging ability (μMol TE 100 g^{-1})
FL62R1 (p<0.0001)	0.3	3.9192 c
	10	5.0395 b
	20	5.1511 ab
	30	5.2507 a
Scotia (p<0.0001)	0.3	4.1845 b
	10	5.2559 a
	20	5.3106 a
	30	5.3977 a
Snowbird (p<0.0001)	0.3	4.0282 c
	10	5.0041 b
	20	5.0885 ab
	30	5.1858 a
13NQW1265 (p<0.0001)	0.3	4.8787 b
	10	5.6624 a
	20	5.7139 a
	30	5.7737 a
Wheat variety × reaction time, p<0.0001 Means with the same letter are not significantly different with 95% confidence limits		

Table 5.3-1. Effect of interaction between wheat variety and mycorrhizal strain on total phenolic content (TPC), sliced by wheat variety (with 95% confidence limits)		
Wheat variety	Mycorrhizal strain	Estimate for TPC (µg ferulic acid equivalent g^{-1})
FL62R1 (p=0.0140)	*F. mosseae*	6.9179 ab
	F. caledonius	6.9232 ab
	R.irregularis	6.8684 ab
	Myke	6.7580 b
	Control	6.9903 a
Scotia (p=0.4990)	*F. mosseae*	6.7657 a
	F. caledonius	6.8130 a
	R.irregularis	6.7050 a
	Myke	6.7856 a
	Control	6.8085 a
Snowbird (p=0.0220)	*F. mosseae*	6.6258 ab
	F. caledonius	6.7236 a
	R.irregularis	6.5840 ab
	Myke	6.5004 b
	Control	6.6496 ab
13NQW1265 (p0.0022)	*F. mosseae*	7.1368 a
	F. caledonius	7.0550 ab
	R.irregularis	7.0267 ab
	Myke	6.9049 b
	Control	6.9033 b
Wheat variety × mycorrhizal strain, p=0.0338 Means with the same letter are not significantly different with 95% confidence limits		

Table 5.3-2. Effect of ineraction between wheat variety and mycorrhizal strain on total phenolic content (TPC), sliced by mycorrhizal strain (with 95% confidence limits)		
Mycorrhizal strain	Wheat variety	Estimate for TPC (μg ferulic acid equivalent g^{-1})
Funneliformis mosseae (DAOM 198274) (p<0.0001)	FL62R1	6.9179 b
	Scotia	6.7657 bc
	Snowbird	6.6258 c
	13NQW1265	7.1368 a
Funneliformis caledonius (DAOM 242686) (p<0.0001)	FL62R1	6.9232 ab
	Scotia	6.8130 bc
	Snowbird	6.7286 c
	13NQW1265	7.0550 a
Rhizophagus irregularis (DAOM 240442) (p<0.0001)	FL62R1	6.8684 ab
	Scotia	6.6705 bc
	Snowbird	6.5840 c
	13NQW1265	7.0267 a
Myke (DAOM 197198) (p<0.0001)	FL62R1	6.7580 a
	Scotia	6.7856 a
	Snowbird	6.5004 b
	13NQW1265	6.9049 a
Control (p<0.0001)	FL62R1	6.9903 a
	Scotia	6.9033 bc
	Snowbird	6.6496 c
	13NQW1265	6.9033 ab
Mycorrhizal strain × wheat variety, p=0.0338 Means with the same letter are not significantly different with 95% confidence limits		

Figure 5.1 Curve plot of DPPH scavenging. TE, Trolox equivalents; *F.caledonius*, *Funneliformis caledonius; F.mosseae*, *Funneliformis mosseae; Myke*, commercial product containing a *Rhizophagus irregularis* strain; *R.irregularis*, *Rhizophagus irregularis*



Connecting text

The previous chapters focused on the influence of arbuscular mycorrhiza on root morphology in different spring wheat varieties under salinity stress and on total phenolic content and antioxidant capacity of selected wheat genotypes. As concluded in previous literature review, mycorrhizal association alleviates salinity stress though multiple ways, including regulation the expression of certain genes. Therefore, identification of genes involved in wheat-mycorrhizal interaction is valuable. Transposon-based gene identification is well documented in plants and has long been used as a genomic tool in gene identification, especially the two-component *Ac*/*Ds* transposon system. Our laboratory has obtained initial wheat *Ac*/*Ds* transposon lines from our collaborator, Dr. Michael Ayliffe, CSIRO Plant Industry Canberra, Australia. In the following chapter 6, we have initiated preliminary screening of these transposon-based wheat mutants, which could be further used to detect genes related to wheat-mycorrhizal association. This study provided material for further gene analysis researches.

Chapter 6

Identification of stable *Ds* transposon mutants in wheat

6.1 Abstract

Wheat plays an particularly important role in human life, being the third most produced staple crops with a global production over 0.7 billion tonnes after maize and rice. Wheat possess numerous desirable agronomic traits such as high yield, stress tolerances and genes related to mutualistic symbiosis in its complex genome, which makes genes exploration and identification extremely important. Transposons are widely existed in plant genomes and are now efficiently used as a functional genomics tool for gene identification, especially the two component *Ac*/*Ds* transposon system. The maize originated *Ac*/*Ds* transposons have been widely used in heterologous species as for gene cloning through insertional mutagenesis and activation tagging. The aim of this study was the identification of stable *Ds* transposon wheat mutants. Forty-eight *Ac*/*Ds* transgenic lines were planted and screened with PCR and thirty-two of them contained both *Ac* and *Ds* element. However, stable *Ds* transposition occurred in only 4 out of those 32 lines with a transposition rate of 12.5%. These 4 putative transposon lines could be further utilized as launching pads for the development of large number of additional genetic mutants which could be used for wheat functional genomic studies, especially genes involved in wheat-mycorrhizal interaction.

6.2 Introduction

Wheat is a staple crop that is of great importance, being as considerable food resource as well as donor for natural antioxidants (Baublis et al., 2000; Onyeneho and Hettiarachchy 1992). The bread wheat has a complex and huge genome. Exploration and identification of agronomically important

genes are of great importance for any crop improvement program. Multiple approaches have been exploited to study functions and interactions of genes, which involves genetic interaction mapping at DNA level; microarrays, SAGE (serial analysis of gene expression) and small RNA sequencing at RNA level; loss of function mutants generated by RNAi and random insertional mutagenesis. Particularly, the transposon-based insertional mutagenesis is one of the most prevalent methods with significant advantages over others in gene tagging approaches. It enables the creation of an intact insertional unit without undesirable modification on integrated locus (Ramachandran and Sundaresan, 2001), a revisable phenotype upon transposon activation and excision (Frøkjaerjensen et al., 2010; Haniford, 2001), a predicable local mutagenesis caused by genetically linked location preference when transposing (Jones et al., 1990; Long et al., 1997; Seki et al., 1999; Zhao et al., 2005) and a controllable occurrence of transposition realized by non-autonomy transposon like *Ds*. Moreover, the transposon-based mutagenesis has the capacity of the generation of both overexpression mutants (activation tagging) and knockout mutants as previous reported by Ayliffe et al. in 2007 in barley.

Transposable elements, which are characterized by their capacity of transposition, are widely spread in eukaryotic genomes, especially abundant in plants. Numerous endogenous transposons have been identified, such as *Ac/Ds*, *En/Spm* and Mutator, etc. (Du et al., 2011; Gierl 1996; Wang et al., 2008).

The maize *Ac*/*Ds* transposable element system was first discovered by Barbara McClintock in 1940s (McClintock, 1947; McClintock, 1948). The *Ac* (*Activator*) encodes a transposase which is necessary for the autonomous transposition of *Ac* element. The *Ds* (*Dissociation*), however, is unable to autonomously transpose within genomes due to the deficiency of self-encoded transposase (Kunze and Starlinger, 1989; Lazarow et al., 2013; Peacock et al., 1987). Therefore,

the *Ds* transposition is controllable based on the appearance of *Ac* element. Compared to single transposable element, these two-component transposons are more prevalent with significant stability, controllability (Fedoroff 1989; Hehl and Baker, 1989) and phenotypic reversion (Lazarow et al., 2013; Lida et al., 1982). Since the first successful application of maize *Ac*/*Ds* in tobacco (*Nicotiana tobaccum*) (Baker et al. 1987; Baker et al., 1986), the *Ac*/*Ds* transposons have been broadly transformed and used as a functional genomic tool in numerous plants, including rice (Chin et al., 1999; Solis et al., 1999; Tatiana et al., 2004), barley (Ayliffe et al., 2007; Singh et al., 2006; Singh et al., 2011), tomato (Biezen et al., 1996; Carter et al., 2013), lettuce (Michelmore, 1994; Yang et al., 1993), etc. In addition, a great deal of genes was discovered with the aid of *Ac*/*Ds* transposon system, ranging from the anther indehiscence1 gene in rice (Zhu et al., 2004), *Curly leaf* in Arabidopsis (Goodrich et al., 1997) to *knotted 1* in corn (Hake et al., 1989), etc. However, researches on transformation and function of *Ac*/*Ds* into wheat for genomic are scarce considering the complexity of wheat genome.

Therefore, based on the hypothesis that transposon-based mutant approach can be useful for gene identification, the main purpose of this research work was the identification of transposon-based wheat mutants, which could be used in the understanding of wheat genome and important genes involved in biotic and abiotic tolerances related plant-microbe interactions, such as wheat-AMF symbiosis.

6.3 Material and methods

6.3.1 Plant materials

Three types of transgenic wheat seeds were provided by Dr. Michael Ayliffe from CSIRO Canberra, Australia, namely T_0 *Ac* transgenic wheat seeds proposed to introduce with *Ac* construct,

T_0 *Ds* transgenic seeds proposed to have *Ds* construct inside and T_0 *Ac*/*Ds* co-bombardment seeds assumed to contain both *Ac* and *Ds* constructs.

---- *Ac* Construct (the *Ubi-transposase* gene)

There are two groups of T_0 *Ac* seeds, namely *Ac* 1 and *Ac* 2, respectively. The *Ac* element has the same construct as previous reported by Ayliffe et al in 2007 (Ayliffe et al., 2007). It is modified with its initial 965 bp replaced by a maize poly-ubiquitin promoter and leaving the remaining sequences reverse complementary to 1093-4689 of the *Ac* wx-m9 sequence as previous reported by Ayliffe et al in 2007 (GenBank K01964). The maize poly-ubiquitin promoter is consisted of three main parts: a 5' leader, the first intron sequence and an *E. coli* omega transcriptional enhancer fragment (Figure 6.1).

----*Ds* construct (the *UbiDs*)

Four groups of T_0 *Ds* transgenic seeds are provided and named as *Ds* 1, *Ds* 2, *Ds* 3 and *Ds* 4, respectively. The *Ds* construct, *UbiDs*, was modified from a maize *Ac* element (*Ac wx-m9*, GenBank K01964) with three external fragments assembled inside. To be specific, the *ubiDs* construct consisted of six fragments. It initialed with a partially 5' *Ac wx-m9* sequence (GenBank K01964, nucleotides 103-373) and followed by a maize poly-ubiquitin promoter (GenBank J944464), an internal *AcXE* fragment (909 bp, nucleotides 2,610–3,519) and another maize poly-ubiqintin promoter (GenBank J944464) and finally ended by with a 222 bp 3'*Ac wx-m9* terminus (nucleotides 4,469–4,691). Additional, an *uidA* gene (*pBI101.2*, CloneTech) was placed immediately adjacent to the *3' Ac wx-m9 terminus*, functioning as a report gene in GUS staining. The full *ubiDs* construct was displayed in figure 6.2 below.

---- T_0 *Ac/Ds* co-transformed seeds

The T_0 *Ac/Ds* co-bombardment seeds were transformed with both *Ac* construct (the *Ubi-transposase* gene) and *Ds* construct (the *UbiDs* element). Forty-eight T_0 *Ac/Ds* wheat seeds were planted in greenhouse under regulated condition: 16-h photoperiod and 22 °C (day)/20 °C (night) temperature regime.

6.3.2 DNA isolation

Genomic DNAs from young leaves of forty-eight T_1 *Ac*/*Ds* co-bombardment wheat lines were extracted following a modified phenol: chloroform method, a protocol reported by Singh et al., 2012.

Leaf tissues were collected in labeled tubes and froze with liquid N_2 before grinding with Tissue Lyser II (Qiagen, Toronto, ON). 700 µl natural extraction buffer and 85 µl 10% SDS (sodium dodecyl sulphate) were then added before tubes were incubated at 65°C for 15 minutes. 200 µl of 5M KOAc (potassium acetate) were then added for DNA precipitation, mixed and centrifuged at 14,000 rpm for 5 minutes after putting on ice for 5-30 minutes. Then, 800 µl of the supernatant was transferred into new tubes and mixed with 450 µl of fresh Phenol: Chloroform (1:1). Tubes were centrifuged for 5 minutes and 750-850 µl of the upper layer (containing DNA) was taken into new tubes slowly. Finally, 700 µl of isopropanol was added to tubes and mixed. DNA precipitates were visible at this stage. Before starting next step, samples were kept in freezer (-20°C).

Samples were centrifuge at 14,000 rpm for 3 minutes before pouring off the supernatant. Washing with 500 µl of 70% ethanol (-20°C), DNA samples were centrifuged and supernatants were slowly removed. This step was repeated twice. Then, ethanol was removed and tubes were dried in vacuum centrifuge. Pellet was re-suspended in 65-70µl of TER (Tris Acetate RNase) and the concentration of DNA was detected either through spectrophotometer (260:280). Finally, DNA stocks were stored at -20^{o}C until further use.

6.3.3 PCR detection of *Ac* and *Ds* elements

The existence of *Ac* (the *Ubi-transposase* gene) and *Ds* (the *UbiDs*) elements and the transposition of *Ds* element in transgenic wheat lines were detected by standard PCR respectively. Primers were designed with Prime 3 according to sequences of *Ac* and *Ds* constructs. To be specific, the primer set Ac3 and Ac5 was designed to detect existence of Ac element. The primer set ubi-3 F_1 and ubi-3 R_1 was specific for existence of *Ds* element and the primer set ubi-uidA F_3 and ubi-uidA R_3 was used to identify transposition event of *Ds* element. The sequence for each primer was listed below in Table 6.1 and the relative binding sites were marked in Figure 6.1 and Figure 6.2 below.

Polymerase chain reaction (PCR) was conducted in a 25 µl reaction mixture: 5µl of reaction buffer (5X), 1µl of forward primer (10µM), 1 µl of reverse primer (10µM), 1 µl of dNTPs (2.5mM), 1µl of genomic DNA，0.25µl of DMSO，0.20 µl of Taq DNA polymerase and 15.55 µl of ddH_2O to adjust the final volume to 25 µl.

The PCR reactions were carried out in the following designed program. Reaction mixtures were initially denatured at 95°C for 3 minutes before entering into cycles which were repeated for 36 times. Each cycle consisted of three steps: DNA was denatured at 95°C for 30s before annealing at 55°C for 30s and followed by DNA extension at 72°C for 60s. A final extension at 72°C for 5 minutes was set after the 36 cycles and the amplification products were kept at 4°C before taking out for storage. The amplification products were detected with 1% agarose gel and the size was confirmed by comparing with a 1Kb DNA ladder (New England, Biolabs).

6.3.4 Flaking sequences identification of *Ds* insertion with inverse PCR (i-PCR)

Genomic DNA of four T1 transgenic lines were digested with TasI (3’ end isolation). Restriction digestion was conducted in a 500µL reaction system (50 µL of 10X reaction buffer, 10 µL of

restriction enzyme, 5 μL of RNase, 10 μL of genomic DNA and 425 μL of sterilized H_2O) and overnight incubation at 37°C was required. Phenol: Chloroform and ammonium acetate (4.4M, PH 5.2) were used to purify the digested DNA before dissolving DNA pellet in 300 μL sterilized water. Overnight ligation was performed in a 500 μL reaction mixture: 300 μL digested DNA, 100 μL 5X ligation buffer, 5 μL T4 H.C ligase and 175 μL sterilized water. The ligation products were purified and diluted before used as DNA templates. Two rounds of PCR reaction were required in a 25 μL reaction mixture, with different sets of primers in each round. Reaction mixture in the first round was made up with 2.5 μL 10X reaction buffer, 0.5 μL forward primer (5-1F), 0.5 μL reward primer (5-1R), 2.5 μL dNTPs (2.5mM), 1.0 μL DNA template (diluted from ligation products), 0.25 μL TaKaRa Ex Taq and 17.75 μL sterilized water to adjust the total volume into 25 μL. Reaction mixture required in the second round was the same with the reaction composition mentioned above in 6.3.3 with a forward primer (5-2F) and reward primer (5-2R). All PCR reactions in the two rounds followed the program descripted below: reaction mixture was initially denatured at 95°C for 5 minutes before entering into 30 cycles. Each cycle was made up of three consecutive steps: 30s DNA denaturation at 94°C, 30s DNA annealing at 56°C and 60s DNA extension at 72°C. Reaction mixture was then extended at 72°C for 10 minutes before kept at 4°C. The amplification product from the first circle was diluted 10X before using as template in the second circle. Sequences for primes sets was present in Table 6.2 below. Amplification products were detected with 0.8% agarose gel and bands were extracted for sequencing (Figure 6.6).

6.3.5 DNA sequencing and bioinformatics analysis

Extracted bands were sent for sequencing at McGill University and Genome Quebec Innovation Centre. The homology of flanking sequences was analyzed with tools provided at NCBI.

6.4 Results

6.4.1 Confirmation of *Ac* and *Ds* in T_1 co-transformed transgenic lines

Primers for *Ac* existence (Ac3 and Ac5) and *Ds* existence (namely ubi-$3F_1$ and ubi-$3R_1$) were used to amplify DNA from forty-eight T_1 *Ac*/*Ds* co-bombardment samples. Amplifications were sequenced and confirmed.

The *Ac* amplification gel result showed that thirty-two out of forty-eight samples had uniform bands with a size smaller than 1kb after amplifying with Ac3 and Ac5 primers (approximately 800bp), which indicated that thirty-two out of forty-eight samples contained *Ac* construct. Similarly, the *Ds* amplification result gel result demonstrated that uniform 1kb bands appeared in thirty-three samples amplified with *Ds* primers (ubi-$3F_1$ and ubi-$3R_1$), proving thirty-three samples contain *Ds* construct.

Moreover, a further comparison of *Ac* amplification gel result and *Ds* amplification gel result illustrated there were thirty-two samples that possessed both *Ac* and *Ds* constructs and those thirty-two samples had the potential of transposition. Figure 6.3 and 6.4 below showed the PCR amplification results with *Ac* and *Ds* primers respectively. Nine samples，namely No.1 to No.9, were involved and taken as examples to show the gel results.

6.4.2 Confirmation of *Ds* transposition

Transposition activities of *Ds* constructs were detected through PCR. Forty-eight DNA samples were amplified with specific transposition primers and the amplification products were then detected in 1% agarose gel (Figure 6.5). This specific transposition primer set was designed based on the *Ds* construct (*ubiDs*) with their relative location marked in Figure 6.2 above. To be specific, the forward primer ubi-uidA F_3 was located in polyubiquitin promoter area and the reward primer ubi-uidA R_3 was designed to complementary with the uidA reporter gene (GUS gene). Therefore,

occurrence of *Ds* transposition was reflected by the disappear of 1 kb amplification products. DNA could be amplified and present in the gel only when *Ds* constructs were not transposed. Otherwise no amplification products will be observed in agarose gel when the *ubiDs* elements were successfully transformed into new locations.

The gel result showed that the 1 kb amplification products were found in twenty-nine out of forty-eight samples. A further comparison between the *Ds* existence (Figure 6.4) and *Ds* transposition results (Figure 6.5) implied that *Ds* transposition may have occurred in 4 putative transposon lines, namely sample No.8, No.10, No.21 and No.47. For example, Figure 6.4 and Figure 6.5 showed the amplification results of nine DNA samples (sample No.1 to No.9). Among them, samples 2, 3, 8, 9 contained *Ds* constructs according to the *Ds* existence results. In the *Ds* transposition results, however, 1 kb amplification bands were present in sample 2, 3, 9 but absent in samples 8, which indicated that *Ds* construct in sample No.8 may have transposed into new location. The other three putative transposon lines were named as sample No. 10, No.21 and No.47. Therefore, a combination of all three PCR results illustrated that thirty-two out of forty-eight samples were found to contain both *Ac* and *Ds* construct and four out of those thirty-two samples were newly transposed lines.

6.4.3 Flanking sequencing identification with i-PCR and bioinformatics analysis

Based on our molecular screening results, there was no lines that contained only *Ds* element. However, there were twenty-eight lines that contained both *Ac* and *Ds* but lost the capacity of transposition. Therefore, four lines were selected within these twenty-eight for i-PCR to locate the *Ds* construct within genome. Using i-PCR, unique *Ds* flanking sequences were obtained in one transposon mutant. The bioinformatics analysis of flanking sequence identified the transposon in

genic region which is mapped on *Triticum aestivum* chromosome 3B. The sequence was an unidentified protein (HG670306.1 and e-value of 2e-135).

6.5 Discussion and conclusion

Transposons are widely spread among eukaryotic genomes, especially within plant genome. Nowadays, it has been broadly used as a genomic tool, especially in the generation of transposon-based insertional mutagenesis. Compared with insertional mutants generated through other approaches, transposon-based insertional mutagenesis has significantly advantage, representing as reversible mutants, in which the disruption and recovery of genes can be achieved by activating and excising transposable elements. Among all available transposable elements, the two-component *Ac*/*Ds* is well documented in other crop species. The modified *Ds* cannot transpose without the transposase encoded by *Ac* element, which made transposition event controllable on demand.

In this study, we first identified the *Ac* and *Ds* bombardment results of 48 T_1 transgenic lines and figured out that 32 out of 48 lines contained both *Ac* and *Ds* elements. Furthermore, another PCR was conducted on all transgenic lines (especially focusing on these 32 *Ac* and *Ds* co-containing lines) with specific *Ds* transposition primers. A combination and comparison between the *Ds* existence results and the *Ds* transposition results screened out 4 putative transposition lines in which *Ds* transposition events may occurred. Therefore, the transposition frequency is 12.5% in wheat. This is comparable with other species such as barley where 9-17% transposition activation was observed (Singh et al., 2006). Our results are preliminary and further confirmation and researches could be required on these forty-eight transgenic lines and more supporting evidence from multiple research approaches should be involved. Firstly, a more precise southern blot would be required to confirm the PCR results. Moreover, further i-PCR are necessary to fine locate both

the initial insertion site of *Ds* element and the new insertion site as the consequence of transposition. Thereafter, the 4 unique lines obtained with our analysis can be used as launching pads to expand this resource further. For example, *Ds* insertions from these lines can be mapped. Unique lines could be used as parent to hybridise with *AC* lines and new F_3 population can be generated. To maximize the benefit of *Ds* tagged mutants, *Ds* insertion sites can be identified in newly generated mutants with i-PCR, Tail-PCR and adapter-PCR methods (Cooper et al., 2004; Singh et al., 2006; Brown et al., 2012; Singh et al., 2012).

Given that transposition activation is higher in heterologous system, introduction of *Ac*/*Ds* into heterologous plants is able to provide abundant mutants, which close links genes with function. Moreover, since the characteristic of transpose locally, *Ac*/*Ds* could also be used in directional research of certain genes, which can be easily achieved by inserting and locating the transposon system near gene of interest. Therefore, this system has great potential for wheat functional genomics (Singh et al., 2006). In the previous chapters, we have discussed how interaction between wheat and mycorrhizal strain affect plant biomass and root architecture under salinity stress. But what genes are responsible for these alternations? These transposon genetic populations could be used to as genomic tools to detect related genes, locate genes and understand function of genes.

Table 6.1 *Ac* specific and *Ds* specific primers for PCR reaction

Primer	5'-3' sequences
Ac forward primer: Ac 3	ACCACCAGCACTGAACGCAGACTC
Ac reward primer: Ac 5	AACCTATTTGATGTTGAGGGATGC
Ds existence forward primer: ubi-3 F_1	CGTCAGACACGTTCTGATTG
Ds existence reward primer: ubi-3 R_1	CATGCAGGGATGAAAGTAGG
Ds transposition forward primer: ubi-uidA F_3	CACTTGTTTGTCGGGTCATC
Ds transposition reward primer: ubi-uidA R_3	GACGTTGCCCGCATAATTAC

Table 6.2 Primers for first and second round of i-PCR reaction

Primer	5'-3' sequences
First round forward primer: 5-1F	ATAACGGTCGGTACGGGA
First round reward primer: 5-1 R	GATTTTGTTAGTTTTATC
Second round forward primer: 5-2F	GGATTTTCCCATCCTACT
Second round reward primer: 5-2R	TTTGTTAGTTTTATCCCG

Figure 6.1 *Ac* construct

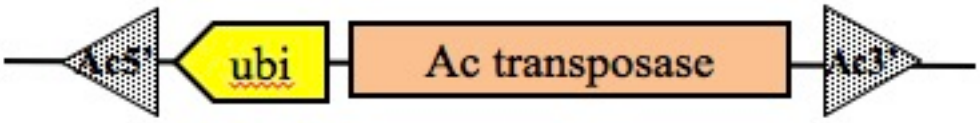


Figure 6.2 *Ds* construct

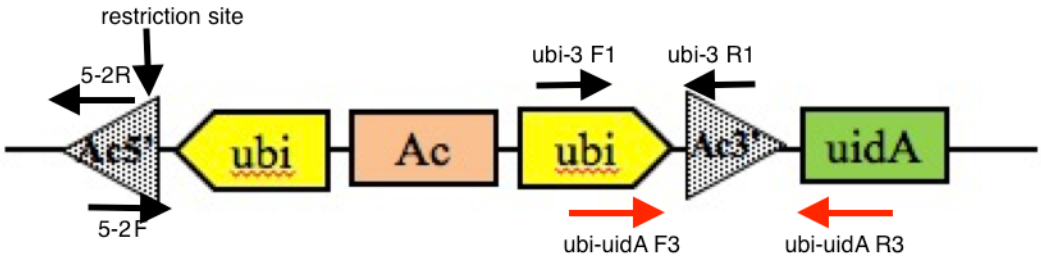
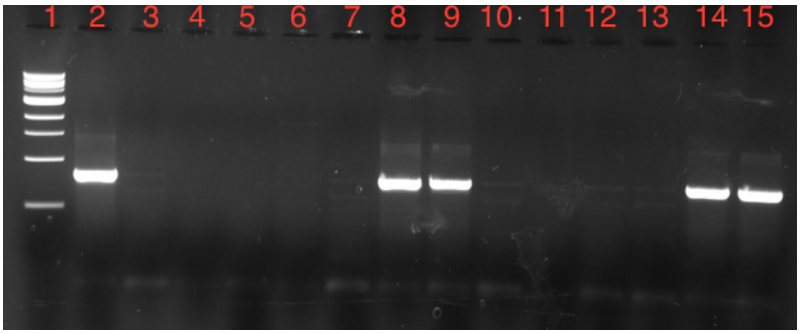
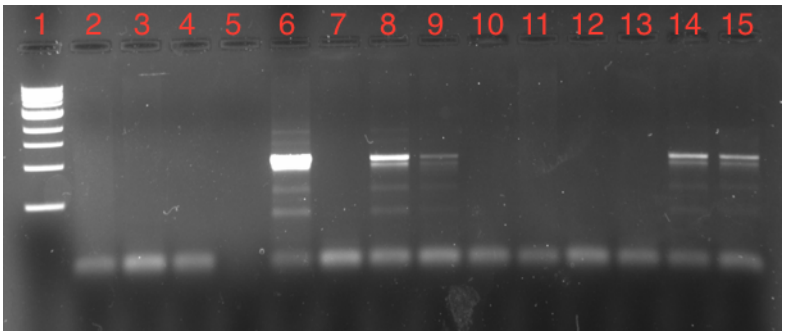


Figure 6.3 PCR analysis of *Ac* element in T_1 transgenic lines. Nine samples were present in gel, starting from lane 7 to lane 15. Bands were found in four samples (2,3,8,9), indicating these four samples contained *Ac*. Bands were sequenced to confirm.



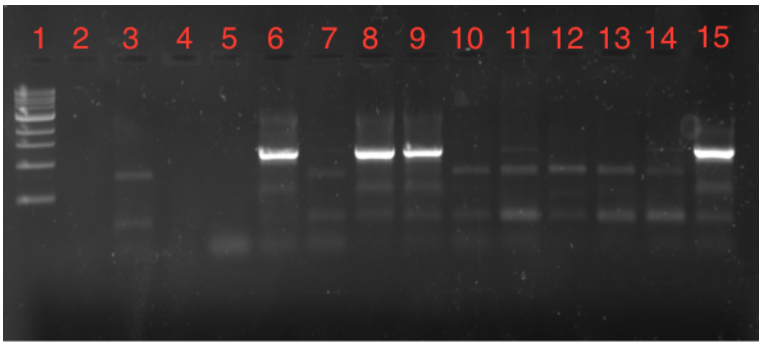
Lane 1: 1 kb DNA ladder
Lane 2: *Ac*/*Ac* (+ve)
Lane 3: *Ds* 4-2 (-ve)
Lane 4: Chinese Spring (-ve)
Lane 5: H_2O (-ve)
Lane 6: empty lane
Lane 7-15: T_1 transgenic line 1-9
Primer F: Ac 3
Primer R: Ac 5

Figure 6.4 PCR analysis of *Ds* element in T_1 transgenic lines. Nine samples were present in gel, starting from lane 7 to lane 15. Bands were found in four samples (2,3,8,9), indicating these four samples contained *Ds*. Bands were sequenced to confirm.



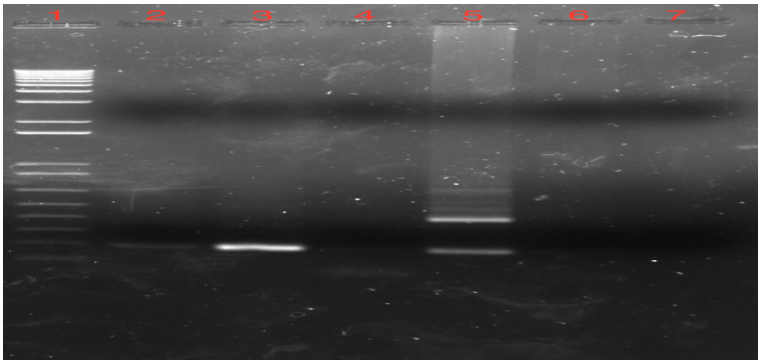
Lane 1: 1 kb DNA ladder
Lane 2: Ac/Ac (-ve)
Lane 3: Chinese Spring (-ve)
Lane 4: H_2O (-ve)
Lane 5: empty lane
Lane 6: *Ds* 4-2 (+ve)
Lane 7-15: T_1 transgenic line 1-9
Primer F: ubi-3F1
Primer R: ubi-3R1

Figure 6.5 PCR analysis of *Ds* transposition events in T_1 transgenic lines. Nine samples were present in gel, starting from lane 7 to lane 15. Bands were found in four samples (2,3,9), indicating *Ds* in these three samples were not transposed. However, band was absent in sample 8, suggesting Ds transposition may occur in sample 8.



Lane 1: 1 kb DNA ladder
Lane 2: *Ac*/*Ac* (-ve)
Lane 3: Chinese Spring (-ve)
Lane 4: H_2O (-ve)
Lane 5: empty lane
Lane 6: *Ds* 4-2 (+ve)
Lane 7-15: T_1 transgenic line 1-9
Primer F: ubi-uidA F_3
Primer R: ubi-uidA R_3

Figure 6.6 i-PCR analysis of *Ds* insertion site in T_1 transgenic lines. Bands below in gel demonstrated the amplification result of second round PCR reaction from 5' end of *ubiDs*



Lane 1: 1 kb DNA ladder
Lane 2: sample 3
Lane 3: sample 5
Lane 4: sample 9
Lane 5: sample 2
Lane 6: Chinese Spring (-ve)
Lane 7: H_2O
Primer F: 5-2F
Primer R: 5-2R

Chapter 7

General Discussion, Conclusion, Future Research and Contributions

7.1 General discussion

7.1.1 The response of spring wheat cultivars to arbuscular mycorrhiza (AM) colonization under salinity stresses

Studies on the responses of wheat varieties to arbuscular mycorrhizal colonization under stress conditions are of great value in multiple aspects. They play important roles in the understanding of potential mechanisms on salinity tolerance, in the selection of preponderant wheat varieties and in the commercialization of more valuable arbuscular mycorrhizal strains for large-scale usage. The purpose of the research was to evaluate the responses of four selected spring wheat varieties to the colonization of four arbuscular mycorrhizal fungi strains under salinity stress. Plant biomasses including grain yield, root fresh and dry weights and root morphological parameters such as root total length, root total volume, root surface area and root average diameter were measured and analyzed through SAS PROC GLIMMIX as indexes for responses evaluation. The results demonstrated that colonization of mycorrhizal strains *Funneliformis mosseae* and *Rhizophagus irregularis* (DAOM 240442) helped mitigate yield losses caused by high saline solutions and helped improve growth and development of root systems as compared to the non-inoculated wheat controls. Salinity, however, had no significant effects on most variables except for grain yield, root surface area and root dry weight, which may be partially due to salinity levels chosen being too low to induce sufficient stress.

A further modified second greenhouse study was conducted to confirm above findings. The best performing AMF strain *Rhizophagus irregularis* and the commercial strain *Myke* were chosen to inoculate on the same four spring wheat varieties as used in preliminary study. Saline stresses were removed, considering the fact that salinity stress had no significant effects on most variables in the previous study. Similar results with first study were acquired, showing *R. irregularis*-inoculated wheat had significantly heavier root fresh and dry weights, a greater root-to-shoot ratio and a stronger root system, as evidenced by longer roots, larger root surface area and smaller root average diameter as compared to non-mycorrhizal controls. The results were consistent with our previous results and therefore successfully supported the findings presented in Chapter 3.

Findings from these two greenhouse studies are consistent with each other, from which we are able to conclude that wheat is advantaged from colonization of the mycorrhizal strain *Rhizophagus irregularis* (DAOM 240442) under both saline and normal conditions. Additionally, the four selected spring wheat varieties were able to survive in slightly saline soil condition (100 mmol/L, which equals to 5.8g/L,) without significant detrimental effects (Brouwer et al., 1985).

7.1.2 Comparison of total phenolic content and antioxidant capacity of mycorrhizal-colonized white, red and purple spring wheat (*Triticum aestivum* L.) genotypes

Mycorrizal colonization benefits plants not only through improving plant yield and altering root architecture but also by modifying antioxidant capacity within host plants, an important mechanism in salinity tolerance. In this study, total phenolic content (TPC) and DPPH scavenging capacity were measured as indicators for the evaluation of antioxidant levels of seeds collected from wheat samples grown in non-saline conditions from the first greenhouse experiment descripted in Chapter 3. The results demonstrated that the purple wheat variety, 13NQW1265, had the highest antioxidant capacity, with the highest value in both the DPPH and TPC assay while

Snowbird wheat had the lowest level of antioxidant capacity. The findings were consistent with the results reported in 2010 by Liu *et al.*, (Liu et al., 2010). Furthermore, mycorrhizal colonization, especially by strain *F. mosseae*, positively increased grain TPC in certain wheat varieties, particularly 13NQW1265, a purple grain line.

These findings will be of great value as new indexes for future wheat-breeding efforts and in the improvement of health-beneficial components in functional foods made from wheat.

7.1.3 Identification of transgenic mutants in wheat

Transposons have been broadly used as a functional genomic tool nowadays, especially in the generation of transposon-based insertional mutagenesis. The objective of the study was the identification of transposon-based wheat mutants and the selection of putative transposition lines. Forth-eight T_1 Ac/Ds co-bombardment transgenic lines were screened with PCR technique and 32 of 48 lines contained both *Ac* and *Ds* elements; *Ds* transposition is thought to be possible in 4 of the lines. Additionally, the original insertion site of *Ds* construct was located in wheat 3B chromosome based on the results from nested inverse PCR technique and bioinformatics analysis.

The results provided evidence for transposition mapping population establishment. We have previously discussed how interaction between wheat and mycorrhizal fungi effected plant biomass and root architecture under saline and normal conditions. However, more molecular information is required to get a deeper understanding of the association and genes responsible for the alternations. These transposon genetic populations could be used as genomic tools to detect related genes, locate genes and understand functions of genes.

7.2 General conclusion

Arbuscular mycorrhizae offer a valuable mutualistic symbiosis in agricultural practice, benefiting plants in both normal and stress conditions. Responses of four selected spring wheat varieties to four mycorrhizal strains under saline stress were evaluated. Based on the analysis results, we found that wheat varieties take advantages from the inoculation of AMF strain *R. irregularis*, represented by an alleviated salt-induced yield loss and stronger root systems (Chapter 3). The results are supported by the subsequent modified second greenhouse experiment, from which same benefits from mycorrhizal strain *R. irregularis* were observed (Chapter 4). Additionally, antioxidant capacity of mycorrhizal inoculated wheat were analyzed and compared. Total phenolic content (TPC) and DPPH scavenging capacity were measured as indexes for antioxidant level evaluation. The results indicate that the purple line 13NQW1265 had the highest TPC value and DPPH scavenging capacity. Mycorrhizal colonization had a non-significant effect on DPPH capacity but inoculation of mycorhizal strain *F. mosseae* positively increased grain TPC in wheat 13NQW1265 (Chapter 5). In addition, a molecular study was described in Chapter 6 for the screening of transposon-based wheat mutants. Molecular screening has been conducted on forty-eight *Ac*/*Ds* transgenic wheat lines and *Ds* transposition was believed to occur in four putative lines. A further nested i-PCR located the *Ds* insertion site on wheat 3B chromosome. These lines could be used to further understand genes responsible for wheat-mycorrhizal interaction and potential mechanisms for salinity tolerance.

In summary, the results from all of the above studies defend our initial hypotheses: arbuscular mycorrhizal symbiosis enhances salinity tolerance in wheat and the responses varies upon the strains of mycorrhiza fungi and wheat varieties. Mycorrhizal colonization in wheat roots enhances plant tolerance towards oxidative stress and colored wheat tends to gain higher antioxidant capacity. Finally, transposon-based mutant approaches can be of great use for wheat gene

identification. The research provides information about wheat-mycorrhizal interactions and produces useful mutants to understand this mutualistic symbiosis better.

7.3 Future research

Future work to be carried out based on the results from above studies include:

1. Stronger saline stresses with higher salt concentrations could be applied in future studies, for a further understanding and confirmation on the interaction between wheat varieties and arbuscular mycorrhizal strains under stress conditions. Giving that wheat is a moderate salt tolerance plant, a higher saline concentration would be considered so as to induce more significant phenotypic alternations. As reported in previous work, significant changes are usually observed in most root morphological parameters when salt solution has a concentration higher than 120 or 180 mmol/L (Tammam et al., 2008). In reference to the studies in Chapters 3 and 4, more valuable information on root architecture alternations could be acquired if a stronger saline stress was applied. Moreover, in the case of the study in Chapter 5, it focused on the effects of the interaction between AMF strains and wheat varieties on oxidant tolerance of wheat grain. A further step would be interesting to examine the interaction between mycorrhizal strains and wheat varieties under salinity stress and a comparison between these two studies may provide a new sight into the role of plant mycorrhization on oxidant tolerance.
2. Exploration on the utilization of *Ac*/*Ds* transposon-based wheat mutants as powerful functional genomics tool in gene identification and functional genomics understanding is of great use. The *Ac*/*Ds* transposons have been widely used by researches in numerous plants, including rice (Chin et al., 1999; Solis et al., 1999; Tatiana et al., 2004), barley (Ayliffe et al., 2007; Singh et al., 2006; Singh et al., 2011), tomato (Biezen et al., 1996;

Carter et al., 2013), and lettuce (Michelmore, 1994; Yang et al., 1993), etc. In the Chapter 6 above, *Ac*/*Ds* co-bombardment transgenic wheat lines were screened and putative *Ds* transposition lines were filtered out, with the *Ds* insertion site located in wheat 3B chromosome. The four unique *Ds* transposition lines can be used as launching pads to expand this resource further. Firstly, unique lines could be used as parent to hybridise with *AC* transgenic lines and generate new F_3 mapping population. Additionally, *Ds* insertion sites can be identified in newly generated mutants to maximize the benefit of *Ds* tagged mutants with i-PCR, Tail-PCR and adapter-PCR methods. Finally, a more important follow-up would be the generation of a transposition mapping population to explore genes involved in wheat-mycorrhizal interactions and salinity tolerance.

7.4 Contribution to science

1. The above studies generated new understanding of the interaction between mycorrhizal fungi and wheat varieties. As reported in Chapter 3 and Chapter 4, wheat responded best to the inoculation of AMF strain *R. irregularis* under both normal and stress conditions. It therefore indicates that the commercialization of mycorrhizal strain *R. irregularis* could be a future research direction. Moreover, from the aspect of wheat variety comparison, results of the above studies have pointed out that wheat Snowbird may not a good choice when higher yield and stronger root systems were taken into consideration.
2. The study descripted in Chapter 5 successfully confirmed the advantages of color wheat in antioxidant tolerance, particularly the purple grain wheat variety. Our results demonstrated that the purple wheat 13NQW1265 had significantly higher antioxidant capacity as evidenced by higher TPC and DPPH scavenging capacity then white and red-seed wheat varieties. Additionally, the purple wheat responded positively to *F. mosseae* inoculation.

The findings pointed out a novel indictor for specific varieties in breeding selection, especially when high natural antioxidant components are taken into consideration.

3. Transposon-based wheat mutants have been identified, which are of great value in functional analysis of wheat-mycorrhizal interaction. To be specific, with the identification of four putative *Ds* transposition wheat lines, further transposition mapping population could be established for the understanding of mechanisms behind and genes responsible for wheat mycorrhization and even a launching pad for future wheat functional genomics researches.

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