DELINEATING ROOT SYSTEM ARCHITECTURE IN RAPESEED/CANOLA (*BRASSICA NAPUS* L.) THROUGH MOLECULAR AND TRANSCRIPTOMIC APPROACHES

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Root system architecture of plant plays a key role in water and nutrient uptake from the soil, provides anchorage and acts as a storage organ. In this current research, we have focused on the molecular and physiological basis of root system variation in canola (*Brassica napus* L.). Genome wide association mappings in a diverse canola germplasm panel with ~37,500 and ~30,200 single nucleotide polymorphism (SNP) markers were conducted under greenhouse and field conditions, respectively. A total of 52 significant SNP markers associated with different root architectural traits were identified in the greenhouse study. Majority of the markers were distributed on five chromosomes, A01, A02, A04, C03 and C06, of *B. napus*. Twenty-two candidate genes related to root growth and development were detected within 50 kbp upstream and downstream of the significant markers. Three of these candidate genes, *P-glycoprotein 6* (*PGP6*), *Tetraspanin 7* (*TET7*) and *ARABIDILLO-2*, were co-localized with three markers on chromosome C03, A01 and A04, respectively. In the field study, 31 significant SNP markers associated with different root traits were detected. A total of 15 root related candidate genes were identified within 100 kbp upstream and downstream of different significant markers. We also analyzed and compared the transcriptomes from the root systems of spring (weak root system) and winter (vigorous root system) growth habits at two different time points, 30 and 60 days. A total of 169,646 transcripts were analyzed, of which, 582 and 555 transcripts were found to be significantly differentially expressed between spring and winter types at 30 and 60 days, respectively. Several cytokinin and gibberellin associated genes and genes sets were found to be upregulated in spring type compared to winter type at 60 days. Cytokinin has proven inhibitory effect on root system architecture in different crops, whereas, gibberellin promote root

elongation but inhibit lateral root growth. Therefore, we suggest that cytokinin and gibberellin may play an important role in root system variation between spring and winter growth habits. Significant marker loci, candidate genes and transcriptome profile identified in this research will assist future research to understand the root system variation in rapeseed/canola.

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DEDICATION

I would like to dedicate this dissertation to my late father **Muhammad Shamsuzzaman**, whose prime concern was to provide me good educational facility despite of having so many limitations, and, to my beloved mother, **Rokhsana Akter** for all the sacrifices she has made to raise me; and inspire me always to go for higher education

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CHAPTER 1: GENERAL INTRODUCTION

Unprecedented increase in agricultural crop production has been achieved since 1930s throughout the world (Myers et al., 2017; Ramankutty et al., 2018). For example, corn yield in United States it increased to ~10 tons/ha since 1935 (US Department of Agriculture, 2017); wheat yield in United Kingdom has increased to 8 tons/ha from ~2 tons/ha since 1930s (Alston et al., 2010). Approximately, eight-fold increase of oil seed crop production has been achieved in oil-seed crops (especially soybean, palm and rapeseed) since 1961 (Ramankutty et al., 2018). However, food demand has also continued to increase over the time due to increasing population. Current yield trend is considered insufficient to meet the future food demand of estimated 9 billion population in 2050 (Haile et al., 2017). In addition, global agriculture is facing other challenges as decreasing cultivable land area, loss of soil fertility, water scarcity or drought, soil salinization, cultivars approaching yield potentials, new pests and diseases etc. (Ray et al., 2012; Ramankutty et al., 2018).

To tackle the current environmental and other issues related to crop production and be prepared for the future needs, plant scientists need to assemble as many tools as possible and follow multiple avenues of crop improvement. Increasing crop production in the last century was mainly focused on using chemicals and fertilizers, expansion of the cultivable lands, improving machineries, and crop improvement through selecting high yielding varieties and modifying plant shoot architecture (mainly yield attributing characteristics) (Koevoets et al., 2016; Myers et al., 2017). However, the belowground root system remained as the “Hidden half” to the plant breeders as a potential traits of crop improvement. Plant root system has multiple functions including uptaking water and nutrient, acting as a storage organ, providing anchorage and

stability to the plant and in many crops root system is the main edible part. Vigorous and deep root system can uptake higher amount of water and nutrient comparing to shallow and less vigorous root system which can contribute in producing higher yield and biomass production (Lynch, 1995, 2013; Marschener, 1998). No wonder, that is why direct positive correlation between root architectural traits and yield has been detected in many crops such as rice (Harada et al., 1984; Morita et al., 1988; Liu et al., 2002; Yang et al., 2012; Steele et al., 2013), wheat (Ehdaie et al., 2010; Wasson et al., 2012; Atta et al., 2013; El Hassouni et al., 2018), maize (Kuchenbuch and Barber, 1987; Hochholdinger et al., 2008; Hammer et al., 2009; Mu et al., 2015), chickpea (Jaganathan et al., 2015; Ramamoorthy et al., 2017), canola (Akhtar et al., 2008; Duan et al., 2009; Rahman and McClean, 2013) etc.

Despite of having clear indication on the positive roles of root system architecture in increasing crop yield, plant breeders were not interested in modifying different root architectural traits in favor of crop yield in last century (Hochholdinger et al., 2008; Zhu et al., 2011; Piñeros et al., 2016). In fact, there are several challenges associated with large scale root phenotypic selection in the field such as plasticity of the root (Malamy, 2005; Pacheco-Villalobos and Hardtke, 2012a; Smith and De Smet, 2012; Koevoets et al., 2016), time and labor intensiveness, destructive to the plots and crops (Nagel et al., 2009; Paez-Garcia et al., 2015) etc. Recent advancement in the field of root phenomics have encouraged plant breeders to breed for high yielding varieties with desirable root characteristics. Many high throughput techniques to phenotype root traits in the field and controlled condition has been developed and applied successfully in different crops so far. Few such examples are shovelomics (Trachsel et al., 2011; Wishart et al., 2013), rhizotron (Singh et al., 2010; Lobet and Draye, 2013), trench profile method (Vansteenkiste et al., 2014), hydroponics (Holloway et al., 2011; Clark et al., 2013),

pouch and wick system, imaging with magnetic resonance imaging (MRI) technology (Schulz et al., 2013) and X-Ray microcomputed tomography (Mairhofer et al., 2013) etc.

In the current research we analyzed the root system architecture variation in canola/rapeseed (*Brassica napus* L.). *B. napus* (AACC, n=19) is an auto-tetraploid oilseed crop evolved through natural hybridization of two other *Brassica* species, *B. rapa* (AA, n=10) and *B. oleracea* (CC, n=9). The name "Canola" stands for "**Canadian Oil Low Acid**" and was developed by lowering the erucic acid and glucosinolate content of traditional *B. napus* in 1970's. Within five decades, canola has become the world second most important edible oilseed crop after soybean (Huang et al., 2016). Global production of canola oil reached nearly 27 million metric tons during 2014-2015 (Elahi et al., 2016). Canola is an important crop in North Dakota state as North Dakota alone produces more than 80% of the total US canola production. Canola has three different growth habits, spring type, winter type and semi-winter type. These three growth habits are morphologically and genetically different from one another and are a great source of genetic diversity in improving canola yield (Kebede et al., 2010; Rahman and Kebede, 2012). Importantly, the spring and winter canola growth habits are distinct in terms of root architectural traits. We utilized this root system variation to identify markers and genomic regions associated with different root architectural traits and candidate genes regulating root architectural traits within those identified genomic regions following genome wide association mapping approach in both field and greenhouse.

Genome wide association mapping or GWAS has widely been used to identify quantitative trait loci (QTL) and molecular markers associated different diseases in human and animals (Begum et al., 2015). In last ten years, GWAS has become highly popular among the plant scientists and breeders as it circumvent the major limitations of traditional bi-parental QTL

(Quantitative Trait Loci) mapping technique and provides some extra benefits (Ersoz et al., 2007a; Zhu et al., 2008; Gómez et al., 2011). GWAS was successfully implemented on many plant species to identify loci associated with different root architectural traits such as rice (Clark et al., 2013a; Courtois et al., 2013), wheat (Sanguineti et al., 2007; Canè et al., 2014), barley (Reinert et al., 2016), maize (Pace et al., 2015), cowpea (Burridge et al., 2017) canola/ *Brassica* sp. (Akhtar et al., 2008; Wang et al., 2017) etc.

Additionally, we analyzed the transcriptomes in canola root system following RNA-seq approach to identify differentially expressed genes, transcription factors and key regulatory pathways associated with root traits between spring and winter types growth habits at different time points. Studying the transcriptomic gene profiles in root system of spring and winter type canola at different time points allowed us to understand regulatory gene networks and complex physiological pathways that shape the root system architecture in canola growth habits. There are several evidences of successful application of transcriptomics techniques in decoding differential gene expression profile in root system of various plant species i.e. *Arabidopsis* (Lan et al., 2012; Vidal et al., 2013; Begara-Morales et al., 2014; Li et al., 2015), *B. napus* (Yong et al., 2014; Dun et al., 2016), rice (Zhai et al., 2013; Yoo et al., 2017), maize (Stelpflug et al., 2016), soybean (Song et al., 2016) etc. A majority of these above-mentioned studies were conducted under contrasting abiotic stress conditions or contrasting nutrient deficit conditions and in the seedling stages. To date, no studies have been conducted to identify complex gene networks and regulatory pathways causing the inherent genetic variation of root system architecture in adult spring and winter canola plants through transcriptomic approach.

The objectives of this study were:

1. To detect the plant growth stages at which the variation of root traits initiates and reaches to maximum.
2. To study the root growth behavior of spring and winter types canola under simulated water stress conditions in greenhouse.
3. To identify significant markers, genomic regions associated with different root architectural traits in both the greenhouse and field conditions through genome wide association mapping approach.
4. To identify root related candidate genes, close to the physical location of the identified molecular markers or genomic regions associated with root traits.
5. To identify key regulatory pathways, differentially expressed genes and transcription factors related to root growth and development through transcriptomics approach.

CHAPTER 2: LITERATURE REVIEW

2.1. Canola/Rapeseed

2.1.1. *Brassica*

The genus *Brassica* is a prominent member of the family Brassicaceae which is one of the most economically important plant family with over 338 genera and over 3700 species. (Cheng et al., 2015; Fahey, 2016). There are 37 diverse species of flowering plants under the genus *Brassica* which are mainly consumed as vegetables worldwide in the form of edible roots, stems, leaves and seeds (oil). Some very important species under the genus *Brassica* are *B. carinata* (Ethiopian mustard), *B. juncea* (Indian mustard), *B. napus* (rapeseed, canola, rutabaga etc.), *B. nigra* (black mustard), *B. oleracea* (kale, cabbage, collard, broccoli, cauliflower, brussels sprout, kohlrabi etc.), *B. rapa* (Chinese cabbage, turnip).

2.1.2. *Brassica* U triangle

Brassica "Triangle of U" is a very popular theory on the development and origin of six most agronomically important *Brassica* spp. described by Nagaharu U (1935). According to this theory, three allotetraploid *Brassica* spp., *B. juncea* (AABB, 2n=36), *B. napus* (AACC, 2n=38 and *B. carinata* (BBCC, 2n=34) were developed by three independent hybridization event between three diploid *Brassica* spp., *B. oleracea* (CC, 2n=18, *B. nigra*, (BB, 2n=16) and *B. rapa* (AA. 2n=20) (Figure1).

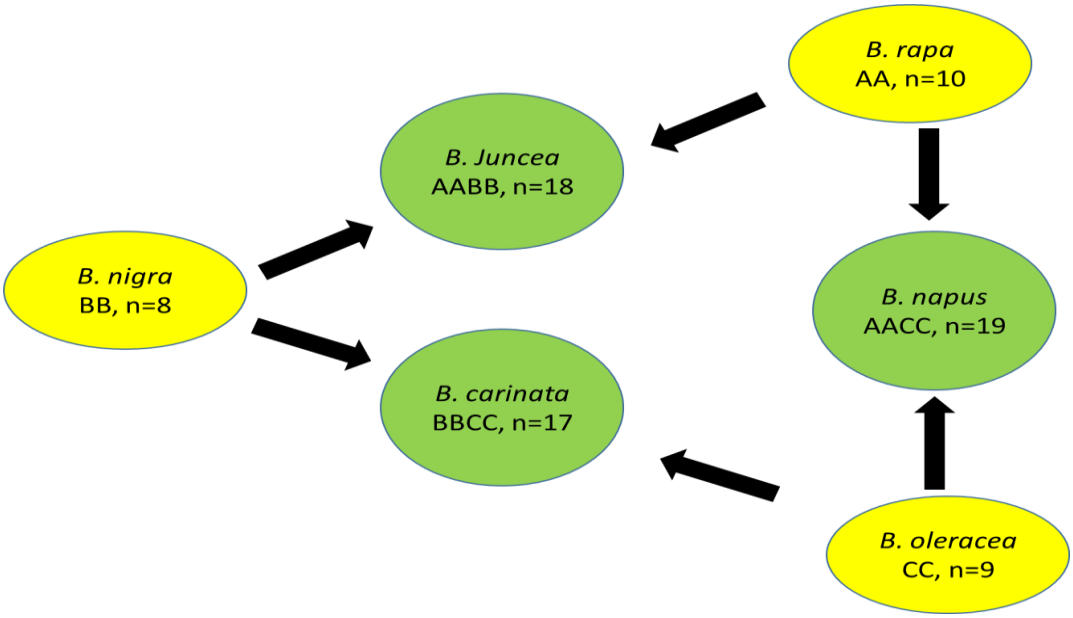

Figure 2.1. Triangle of U showing the genetic relationship among the six *Brassica* spp.

2.1.3. *Brassica napus*

B. napus is an allotetraploid species containing both A genome and C genome. It was developed through recurrent natural hybridization between two other diploid species *B. rapa* and *B. oleracea*. *B. rapa* contributed the A genome and *B. oleracea* contributed the C genome of the *B. napus* species. It is speculated that, the spontaneous natural hybridization that formed *B. napus,* took place in the Mediterranean region of south western European regions (Cruz et al., 2007). It is commonly known as rapeseed, swede rape, argentine rape, oil rape, oilseed rape etc.

2.1.4. Origin and domestication of *B. napus*

Genus *Brassica* is distantly related to *Arabidopsis* and believed to be originated from a common ancestor approximately 20 million years ago (Yang et al., 1999; Wang et al., 2011b). Species *B. napus* was originated within last 10,000 years (Wang et al., 2011b). The center of origin of *B. rapa* is the highlands near the Mediterranean sea from where it spread towards Eastern Europe, Germany and Scandinavia (Nishi, 1980; Tsunoda, 1980). On the other hand, the

B. oleracea is believed to be originated in southern and Western Europe. As *B. rapa* were disseminating from Mediterranean to the western or northern European region, some scientists believe that *B. napus* might be originated anywhere between these places wherever *B. rapa* and *B. oleracea* wild types got chance to grow together (Tsunoda, 1980). Some other theories described the possibilities of *B. napus* formation in some other places where *B. rapa* and *B. oleracea* grow wild and naturalized wild form of *B. napus* were found (Rakow, 2004). In terms of domestication, *B. napus* is a relatively young species with only 400 years of domestication history.

2.1.5. Taxonomy and botany

B. napus is an annual or biennial flowering plant under the family Brassicaceae. Its stem is erect, slender and free branching with waxy leaves. The flowers are bisexual and form on the main and axillary branch. Flowers consist of four sepals, four petals, six stamens and a pistil of two carpels. The ovary is superior type and positioned above the receptacle (Gulden et al., 2008). *B. napus* is mainly a self-pollinating crop. However, 12-47% cross pollination can occur under field condition due to insects, wind or physical contact (Williams et al., 1986; Becker et al., 1992). The fruit is a linear two-celled silique or pod containing seeds inside (Bilay, 1976; Gulden et al., 2008)

2.1.6. Development of canola/rapeseed

Canola or Rapeseed do not refer to a single species. The term "Rapeseed" is used for the oilseeds from both *B. napus* and *B. rapa* species. Canola is a particular type of rapeseed which contains less than 2% erucic acid and less than 30 µmol glucosinolate per gram of air-dried oil-free meal. The term "Canola" is derived from "***Ca***nadian ***O***il ***L***ow ***A***cid" and registered in

Canada in 1978. Now in Canada, United States and Australia, "Canola" is officially defined as the rapeseed oil that contains less than 2% erucic acid and less than 30 µmol glucosinolates per gram of air-dried oil-free meal. In Europe, it is generally known as rapeseed.

Oilseed rape including *B. napus* was growing from ancient times and used as edible oil and lamp fuel. During the industrialization in the early last century, *B. napus* oil was used as lubricant due to its high erucic acid content. During the Second World War, the use of rapeseed oil as lubricant in war and in merchant ships increased significantly. The demand was so high that the plant breeders were focused to develop high erucic acid and high glucosinolate rapeseed varieties. In 1954, the first registered high erucic acid and high glucosinolate variety "Golden' were released in Canada. However, the demand of rapeseed oil as lubricant decreased and plant breeders' focus was shifted to develop a healthy alternative source of edible oil from rapeseed by lowering the erucic acid and glucosinolate content (Rakow, 2004). The first naturally occurring low erucic acid *B. rapa* line "Liho" was discovered in 1963 in University of Manitoba (Stefansson and Hougen, 1964). Three years later in 1966, the first commercially developed low erucic acid *B. napus* line "Oro" was released in Canada. The first naturally occurring low glucosinolate *B. napus* line was "Bronowski" was found in Poland in 1967. Discovery of both naturally occurring low erucic acid and low glucosinolate lines paved the path to develop double low rapeseed variety through plant breeding. Finally, the first double low *B. napus* line named "Tower" was developed by Dr. Baldur Stefansson in 1974 at University of Manitoba (Brown et al 2008).

2.1.7. Canola oil health benefit

Canola oil is very popular for its nutritional qualities. Canola Council of Canada (2017) summarized the canola oil nutritional quality as "high in good fats, lowest in bad fats, no

cholesterol and good source of Vitamin E". Canola oil contains "good for health" Omega-6 fatty (Alpha-linolenic acid) and Omega-3 fatty acid (Linoleic acid) in a ratio of 2:1, which is nutritionally ideal. These unsaturated fatty acids help to reduce the bad cholesterol in human body and lower the risk of heart diseases. In addition, canola contains lowest "bad for health" saturated fatty acid among all the vegetable oils and no trans fats at all. The Low Erucic Acid Rapeseed (LEAR) oil with less than 5% erucic acid and low glucosinolates was introduced in Europe as an edible oil in 1977 (Przybylski et al., 2005; Lin et al., 2013). United States Food and Drug Administration certified canola oil "generally recognized as safe" (GRAS) status in 1985. According to Lin et al (2013), canola oil can be regarded as the healthiest edible oil among all, in terms of its biological functions and its ability to reduce the disease related risks.

2.2. Root system architecture in plants

2.2.1. Root system architecture

The structure, shape and spatial arrangement of the root system altogether in the soil is referred as the root system architecture (RSA) of a plant (de Dorlodot et al., 2007; Koevoets et al., 2016). RSA of a crop is composed of different individual root traits i.e. root length, primary root branches, lateral root branches, root angel, root diameter, root mass, root hair etc. The root system of crop plants are mainly of two types, tap root system and fibrous root system (Comas et al., 2013).

In a taproot system, the primary root that starts from radicle and form the main root is called the taproot. Taproot length is an important characteristics in dicot plants which determines the capability of the plant to explore water and nutrient from the deeper soil zone. It is crucial in response to drought stress where plants survival and retaining productivity largely depends on how deep a root system plant a can produce to adapt the situation (Kong et al., 2014). In addition

to moisture, longer tap roots help plants extract nutrients like nitrogen which usually leached down from the surface soil to deep soil zone (Wasson et al., 2012; Paez-Garcia et al., 2015). Therefore, greater root length and greater rooting depth is important under water limiting condition in soil for yield stabilization (Ludlow and Muchow, 1990).

Primary and secondary root branches grown from the taproot are together termed as the lateral roots and together provide overall shape and density of the root system. In *Arabidopsis* and *B. rapa*, lateral roots are important for water and nutrient uptake during vegetative and reproductive stage (Smith and De Smet, 2012). Root angle is one of the main characteristics of "steep-cheap-deep" root system described by Lynch, (2013). Root system with lower angle will not explore horizontal soil layer, instead will grow vertically along with taproot. This will result less competition with neighboring plants, less energy expenditure and exploration of water and nutrients from deeper soil zone. Higher root and/or taproot diameter represents vigorous root system and helpful for plant to explore water from deeper soil zone. Taproot with higher diameter is linked with higher total xylem area in the root (Burridge et al., 2017) which indicates that it may have higher water and nutrient transport efficiency from root to shoots. In the fibrous root system, several slender adventitious roots are grown downward and outward from the stem forming a dense fine root system mass.

2.2.2. Role and function of root system architecture in plants

Plant root system, referred as 'the hidden half", plays multiple roles in plant growth and development. It is the root system by which plants get access to the soil moisture and nutrients and uptake them. In addition, it gives anchorage and mechanical support to the plant. Root system can also act as storage organ for carbohydrate and is an active site of producing different phytohormones needed for plant growth and development (de Dorlodot et al., 2007). Nodules in

the legume root system can fix nitrogen from the environment with the help of nitrogen-fixing bacteria. Root system from some plants directly serve as the source of human food consumption i.e. radish, beets, carrots, ginger etc.(Zhu et al., 2011). Root system is the first organ which responds with the change rhizosphere and help plants to sense different abiotic and biotic stresses.

2.2.3. Correlation between root system architecture and yield in different crops

Water use, water use efficiency and harvest index are the three major components that determine the crop yield (Passioura, 1977; Turner et al., 2001; Ye et al., 2018). Amount of yield decrease is positively correlated with the water reduction in the soil, and higher water uptake capability of plant clearly improve drought tolerance with improved productivity in water limiting condition (Passioura, 1977; Ye et al., 2018). Root system in plants plays the key role in uptaking water and nutrient resources from the soil. A healthy root system is essential for optimal plant growth; and optimal plant growth can significantly boost the yield (Marschener, 1998). Therefore, variation in root architectural traits and differences in their spatial distribution in the soil profile have substantial impact on water and nutrient uptake capability of plant and their ability to adjust with abiotic stresses to maintain the productivity (Lynch, 2007; Zhu et al., 2011; Piñeros et al., 2016).

Different root architectural traits are positively correlated with yield in different crops. Root number and root length density are directly correlated with rice (*Oryza sativa*) grain yield (Harada et al., 1984; Morita et al., 1988). Additionally, some mathematical models showed the correlation between root biomass and rice grain yield and root oxidation activity and rice grain yield (Liu et al., 2002; Yang et al., 2012). In wheat, Ehdaie et al., (2010) found significant positive correlation of plant nutrient uptake and grain yield with root biomass and suggested to

developing wheat genotypes with superior root characteristics in breeding programs for higher grain yield. Wasson et al., (2012) proposed that wheat varieties with deep root system will have higher root density at deep soil zone than the surface soil zone and will have increased yield in rain-fed system where crops rely on water from deeper soil zone. Additionally, positive relationships were identified between different root traits (root length, root length density and root diameter) with aboveground crops traits including yield in both well-watered and water limiting condition (Atta et al., 2013). However, relationships were stronger in well-watered condition comparing to water limited condition. El Hassouni et al., (2018) showed that 37-38% yield advantage can be obtained with durum wheat varieties with deeper root system under water limiting condition. In maize (*Zea mays* L.), Kuchenbuch and Barber, (1987) showed that, yearly variation of rooting depth and distribution in the soil may cause the variation in the yield in different years. Crop modeling approach on historical increase of maize yield in United States revealed that change in root system architecture and water uptake capability had a significant effect on biomass production and yield increase (Hammer et al., 2009). Root hair elongation in maize can significantly improve yield (Hochholdinger et al., 2008). Genetic improvement of maize root system growth helps maize plants in uptaking higher amount of nitrogen during post-silking stage resulted increase in maize yield (Mu et al., 2015). Under water limiting condition root length density, total root dry weight and deep root dry weight were positively correlated with chickpea yield (Jaganathan et al., 2015; Ramamoorthy et al., 2017). Shallow root system under phosphorus limiting condition is positively correlated with improved field performance in Soybean (*Glycine max* L.) and common bean (*Phaseolus vulgaris*) (Zhao, 2004; Ho et al., 2005; Miguel et al., 2013).

2.2.4. Correlation between root system architecture and yield in *Brassica* species

In rapeseed/canola, positive correlation has been established between root length and shoot phosphorus content under phosphorus limiting soil (Solaiman et al., 2007; Hammond et al., 2009) indicating role of root system in higher phosphorus uptake efficiency that may lead to the improved field performance. Later, Duan et al., (2009) found that lateral root length is positively correlated with phosphorus uptake and yield in canola. Root biomass in canola is directly correlated with biomass accumulation which also may lead to increased productivity in *B. napus* (Akhtar et al., 2008; Duan et al., 2009) and white mustard (*Sinapis alba* L.) (Hajzler et al., 2018). Koscielny and Gulden, (2012) suggested that root length in the early growth stages (one-to two-leaf and three-to four-leaf stages) is one of the best indicator of seed yield. Rahman and McClean, (2013) showed significant and positive correlations between different root traits and agronomic characters of canola (*B. napus* L.), such as, days to flowering vs. root length, days to flowering vs. dry root weight, pods per plant vs. root length, root length vs. dry stem weight, root length vs. dry root weight, root length vs. seed yield, dry stem weight vs. dry root weight.

2.3. Breeding for root architectural traits

2.3.1. Selection for root traits and challenges

Selection for the bellow ground root architectural traits in traditional breeding methods was not a popular strategy to improve the crop yield in last century (Hochholdinger et al., 2008; Zhu et al., 2011; Piñeros et al., 2016). Rather application of different chemicals and fertilizers, increasing the planting density and different management practices were the most popular strategy. Plant breeders, in last few decades, started to realize that modifying shoot architectural traits and yield attributing characteristics is very effective for increasing yield (Koevoets et al., 2016). However, root traits remains as the most under-utilized source of crop improvement

(Herder et al., 2010; Wachsman et al., 2015). This is because of several challenges associated with large scale selection for the root traits following traditional breeding methods. One of the major challenges is the plasticity of the plant root system. Root system architecture is plastic in nature and tends to modify root apparatus according to soil micro-environment, soil water and nutrient condition (Malamy, 2005; Pacheco-Villalobos and Hardtke, 2012b; Smith and De Smet, 2012; Koevoets et al., 2016). This leads to the variation in root system architecture in response to environment. For example, primary root number and length in *Arabidopsis* remain constant in response to a wide variety of nitrogen level in the soil while the length of lateral roots are actually stimulated under low nitrogen condition (Gruber et al., 2013; Kong et al., 2014). On the other hand, both primary and lateral root growth is inhibited but lateral root density is increased in *Arabidopsis* and rice in phosphorus deficient soil (Desnos, 2008; Gruber et al., 2013). Doussan et al., (2009) suggested that nutrient uptake capability actually depends on the plants' ability to modify its root architectural traits according to the soil condition.

Other constraints regarding selection of root traits includes time and labor intensiveness. Incredible amount of time and labor need to be employed to select lines phenotypically with improved root characteristics in the field condition. Moreover, unlike the aboveground plant parts, root phenotyping methods are destructive in nature. This means, it is impossible to measure root traits without destructing the plants and field plots, therefore, selection for higher yielding lines with superior root characteristics is very difficult (Nagel et al., 2009; Paez-Garcia et al., 2015).

2.3.2. High throughput phenotyping for root traits

High throughput phenotyping procedures allow plant scientists to phenotype plant traits in easy, rapid and cost-effective manner with high accuracy. Considering several limitations of

phenotyping root system in a large scale, high throughput root system phenotyping is an effective solution toward root related research. To date, several high throughput root system phenotyping procedures were developed and successfully implanted to phenotype crop root system. Few examples are shovelomics in maize and potato (Trachsel et al., 2011; Wishart et al., 2013), rhizotron in maize and sorghum (Singh et al., 2010; Lobet and Draye, 2013), trench profile method in cauliflower and leek (Vansteenkiste et al., 2014), hydroponics in corn and rice (Holloway et al., 2011; Clark et al., 2013b), transparent media in rice and soybean (Fang et al., 2009; Topp et al., 2013), pouch and wick system in canola (Thomas et al., 2016), imaging with magnetic resonance imaging (MRI) technology (Schulz et al., 2013) and X-Ray microcomputed tomography (Mairhofer et al., 2013) etc. Among these, MRI and X-ray microcomputed tomography is nondestructive methods and able to capture seedling root images directly from the pot. Beside these, 2-D and 3-D root imaging were implemented for several of the above-mentioned high throughput phenotyping methods enabling more accurate and rapid root phenotyping.

2.3.3. Marker assisted selection/breeding for root architectural traits

Plant breeding has two major avenues, traditional or classical plant breeding and molecular plant breeding. Classical plant breeding employs traditional selection procedures and breeding methodologies to improve a crop with desired traits. Molecular plant breeding, on the other hand, applies molecular biology, molecular markers and biotechnology to improve for crop improvement (He et al., 2014). Marker assisted selection (MAS) is one of the major approaches under molecular plant breeding techniques which allows molecular markers for indirect selection of desirable phenotypic traits. MAS has been popular among the plant breeders due to several benefits over classical breeding methods. These includes early generation large scale selection,

reducing breeding cycle, reducing cost and labor, eliminating environment influences during selection (He et al., 2014; Chitwood et al., 2016). Rapid advancement of the molecular marker technologies further popularized marker assisted selection in last few years.

Molecular markers are now an integral part of plant molecular breeding and genetics and used for a range of purposes such as segregation analysis, genetic mapping, phylogenetic and diversity analysis, gene tagging, plant disease diagnostics and marker assisted selection (Kumar et al., 2012; He et al., 2014; Chitwood et al., 2016). DNA molecular markers can be of two types, hybridization-based markers, PCR-based markers and sequenced based markers. Hybridization based Restriction Fragment Length Polymorphisms (RFLP) was the first DNA based molecular marker used for genotyping and creating genetic linkage map (Botstein et al., 1980). Later several PCR based molecular markers, Random Amplified Polymorphic DNA (RAPD) (Williams et al., 1990), Simple Sequence Repeats (SSR) (Hearne et al., 1992), sequence characterized amplified region (SCAR) (Paran and Michelmore, 1993), cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel, 1993), Sequence Tagged Sites (STS) (Fukuoka et al., 1994), Amplified Fragment Length Polymorphism (AFLP) (Vos et al., 1995), Sequence Related Amplified Polymorphism (SRAP) (Li and Quiros, 2001) etc. were developed. Sequenced based markers are Single Nucleotide Polymorphism (SNP) (Marth et al., 1999), Indels etc. Among all the molecular markers, SNPs are now the most popular molecular marker due to its high abundance in the genome and low cost discovery (Zhu et al., 2008; He et al., 2014).

Therefore, availability of high throughput phenotyping, genotyping and molecular marker techniques enable plant breeders to perform MAS through identify molecular markers tagged with gene of interest and quantitative trait loci (QTL) associated with different complex traits.

Considering complexity of root architectural traits, MAS can be very good alternative of traditional breeding method to develop varieties with desired root traits (Courtois et al., 2013). QTL associated with different root traits were discovered in different crops. Several QTL associated with root length and thickness in rice were identified (Steele et al., 2013) and incorporated those in an *Oryza indica* variety through MAS. There are few other examples of identification and successful introgression of root associated QTL in different crops, i.e. wheat (Placido et al., 2013), chickpea (Varshney et al., 2013) etc. Identification of QTL associated with different root traits in *Brassica* species were conducted mostly in response to nutrient deficiency or drought condition (Arifuzzaman et al., 2016). Several QTL were identified for root vigor (Arifuzzaman et al., 2016), root dry weight and primary root number under boron deficiency (Shi et al., 2011), root mass under drought condition (Fletcher et al., 2015) in *B. napus* and taproot characteristics in *B. rapa* (Lu et al., 2008).

2.3.4. Genome wide association mapping

Identification of molecular markers and QTL associated with a trait of interest is the main prerequisite for successful implementation of MAS (Collard et al., 2005). Genome wide association study (GWAS) is a powerful gene mapping strategy that have been developed and used to identify QTL associated with quantitative traits in human and animals (Begum et al., 2015). Later it become very popular in identifying QTL associated with quantitative traits in plants (Zhu et al., 2008). There are several benefits of GWAS mapping strategy over traditional bi-parental mapping. In bi-parental mapping, required mapping population is created from two extreme opposite parents, therefore, it takes into account only the recombination events occurred between the two parents which is relatively low. Due to low recombination events, it is very difficult to identify closely linked markers for MAS (Zhao et al., 2014). In addition, bi-parental

populations have limited number of polymorphic loci with which minor QTL are not detected. In contrast, GWAS requires a diverse germplasm panel consisting of several hundreds of unrelated genotypes from diverse sources and origins (Zhu et al., 2008; Zhao et al., 2014). Due to using large numbers of diverse genotypes, GWAS mapping strategy can capture all the historical recombination events occurred in the mapping panel which results high resolution genetic map (Ersoz et al., 2007b; Gómez et al., 2011). The basis of GWAS is the non-random association of alleles at different loci known as linkage disequilibrium (LD) (Zhu et al., 2008; Zhao et al., 2014). A high density molecular marker panel (mostly SNPs are used) with a good coverage of the genome is essential to detect the recombination breakpoints in the population effectively (Flint-Garcia et al., 2003; Begum et al., 2015). Association mapping has some limitations too. One of the major limitation is detection of false positive association due to population structure and relatedness between the genotypes within the population (Celik et al., 2016). False positive associations can be minimized by taking account of the population structure and relatedness in the population during the analysis. Population structure (Q), kinship matrix (K) and principle component analysis can be incorporated in statistical models like general linear model (GLM) and mixed linear model (Price et al., 2006; Yu et al., 2006; Bradbury et al., 2007).

2.3.5. Genome wide association mapping on root architectural traits in different crops

To date, not many genome wide association mapping have been conducted to identify molecular markers and loci associated with different root architectural traits in comparison to shoot architectural and other traits in different crops. Courtois et al., (2013) conducted GWAS on Japonica rice panel consisted of 167 genotypes and detected 51 unique loci for maximum root length, deep root biomass, root biomass; root to shoot ratio, root cone angle. With GWAS, Clark et al., (2013) detected eight QTL for primary root growth and total root growth that were co-

localized with previously identified QTL with bi-parental mapping. In durum wheat, 15 QTL associated with primary root angle, total root dry weight, spread of root angle, total length of seminal root and number of seminal roots were identified by Sanguineti et al., (2007) with a SSR based GWAS study on 57 genotypes. Canè et al., (2014) studied root traits variation in a durum wheat germplasm panel consists of 183 genotypes and conducted GWAS. They identified 48 QTL for different root architectural traits. Further, they compared the root associated QTL with the QTL identified for different agronomic and yield attributing traits using the same panel and detected 15 overlapping QTL for root and shoot traits. In barley, 11 putative QTL associated with root dry weight, root length and root-shoot ratio were identified in a GWAS mapping study with 179 genotypes and 58,692 SNPs. In maize, total 268 marker-trait association were detected for 22 different root traits in a panel of 319 genotypes by implementing both GLM and MLM in the GWAS (Pace et al., 2015). In cowpea, root traits were phenotyped by following shovelomics approach and 11 significant QTL were identified through genome wide association study (Burridge et al., 2017). They also identified additional 21 QTL through an image-based phenotyping system for different root traits.

Very limited examples of implementing GWAS to identify markers linked with root traits in *Brassica* sp. has been completed to date. Wang et al., (2017) performed GWAS for different root traits with a panel of 405 *B. napus* lines and detected 285 SNPs associated with different root traits in contrasting phosphorus level. Akhtar et al., (2008) identified one QTL for root diameter and root length in *Brassica juncea* through GWAS with a small panel consisted of only 48 genotypes.

2.4. Differential gene expression study

2.4.1. Transcriptomics

A complete set of transcripts or RNA-profile in a cell in a specific physiological condition or developmental stage is known as transcriptome (Wang et al., 2009). Transcriptomics is the study of these transcripts or RNA-profile at a specified condition (Pandit et al., 2018). Transcriptomics is an important method to understand the functional elements of the genome and revealing the molecular constituents of cells and tissues (Wang et al., 2009). Transcriptomics methods such as serial analysis of gene expression (SAGE), cap analysis of gene expression (CAGE) and microarray allowed plant scientists to study the transcriptional changes in contrasting treatments throughout the last few decades (Grunstein and Hogness, 1975; Yamamoto et al., 2001; Samolski et al., 2009; Pandit et al., 2018). More recent, RNA-sequencing technology has become popular as it circumvents the limitations of hybridization based microarrays techniques and allows studying differential expression of the transcript in a more sophisticated manner (Wilhelm and Landry, 2009; Zhang et al., 2014; Yu and Lin, 2016). RNA-seq method provides complete information about the transcripts rather than just measuring relative gene expression and more sensitive to the low-expression transcripts (Zhang et al., 2014). In addition to monitoring global gene expression changes, RNA-seq has been adopted to detect candidate genes through comparative analysis of transcripts under contrasting treatments (Alvarez et al., 2015; Song et al., 2017).

The first step of transcriptomics through RNA-seq is collecting total RNA from the targeted tissue. The total RNA is then converted to cDNA libraries with specific adaptors attached with cDNA fragments at both ends. cDNA libraries are then sequenced through high throughput sequencing techniques available to obtain short one end or pair end reads. The size of

the reads varies with different sequencing techniques. The single end or pair end reads are then aligned with the reference genome of the species to identify the transcripts with their level of expression (reviewed by Wang et al. (2009)

2.4.2. Transcriptomics on root architectural traits in *Brassica* and other crops

Several transcriptomics studies were conducted to identify the complex regulatory pathways and gene networks involved in growth and development of different root traits. Jones et al., (2006) analyzed transcriptomes from *Arabidopsis* roots collected from wild type plants (with root hair) and mutant plant (without root hair) and compare the transcriptomes. From significantly differentially expressed genes list, they analyzed 159 T-DNA insertion lines and finally identified six new genes involved in root hair morphogenesis through reverse genetics. Transcriptomes from radish roots collected at early and late seedling stage were compared by Wang et al., (2012). They suggested that starch and sucrose metabolism and phenylpropanoid biosynthesis may be the dominant metabolic events during tuberous root formation in radish. Stelpflug et al., (2016) identify 1,110 differentially expressed transcription factor genes across longitudinal (four zones) and radial gradients (cortical parenchyma and stele) of the maize primary root, many of which are known for regulating root growth and development. Recently, Rodriguez-Alonso et al., (2018) compared root transcriptomes of *Pachycereus pringlei* (member of Cactaceae) at three developmental stages to unravel the key regulatory paths involved in root apical meristem (RAM) exhaustion and determinate primary root which is a common feature of many Cactaceae members. They identified many putative orthologues of *Arabidopsis* hormone signaling and metabolic pathway related genes. They suggested that specific transcriptional programming at root apex of *Pachycereus pringlei* at specific time points/developmental stage. Comparative transcriptomics were conducted to identify the genes and regulatory pathways

responsible for primary root growth in *B. napus* by Dun et al., (2016). They divided primary root growth into two distinct group short and long and compare the transcriptomes for extremely opposite four lines falls under two groups. They identified 20 differentially expressed phytohormone related genes that may have potential roles in primary root growth differences in *B. napus*. In soybean, identified several differentially expressed genes involved in hormone (Auxin/Ethylene), carbohydrate, and cell wall-related metabolism (XTH/lipid/flavonoids/lignin) pathways in soybean primary roots under three contrasting water deficient stress level, very mild stress, mild stress and severe stress. Jia et al. (2017) compared transcriptomes from roots of club root susceptible and resistance Chinese cabbage (*Brassica rapa ssp. pekinesis*) and found that phytohormone auxin and cytokine related genes were upregulated in susceptible genotypes. Cheng et al., (2016) treated *B. napus* seedling with nanomaterial graphene oxide and found an inhibitory effect of graphene oxide on root growth. Analyzing transcriptomes from treated and control seedlings they concluded that graphene oxide regulates root growth and development through modulating ABA and IBA concentration.

2.5. Physiology of root development

2.5.1 Cellular organization of root system

A growing *Arabidopsis* root tip can be divided into three zones, meristematic region, elongation zone and differentiation zone. The meristematic region is further divided into two zones, apical meristem and basal meristem. At the top of the apical meristem zone, there are a type of mitotically inactive cells forming the quiescent center. This quiescent center promotes the continuous division of the neighboring cells known as stem cell initials which leads formation of new cells (Overvoorde et al., 2010). AP2 types of transcription factors encoded by *PLETHORA 1* (*PLT1*) and *PLETHORA 2* (*PLT2*) genes are responsible for quiescent center speciation and stem

cell activity (Aida et al., 2004). There are four types of stem cell initials give rise to different type of cell layers, epidermis, cortex, endodermis, pericycle, stele, lateral root cap and columella. The epidermis and lateral root cap are the outer layers of the root system and root cap, respectively, and are formed from the division of epidermal/lateral root cap initials. Columella cells occupying the central and tip portion of the root cap are produced from columella initials. Cortex and endodermis are formed from the division of cortex/endodermis initials. The vascular initials divide and contribute to the formation of vascular tissues and pericycle. As a result of continuous division of the four types of initials, the older cells are displaced from the apical meristem zone and transferred to basal meristem. Here, the rate of the cell division is slows down, instead, the cells starts elongating (De Smet et al., 2007; Overvoorde et al., 2010). Eventually they move to the elongation zone where the cell division stops, instead cells starts to elongate in full scale. When cells reach their ideal size and shape, the elongation process ceased and root hairs start to emerge from the epidermis (Dolan et al., 1994). In the next step, elongated and matured cells move towards differentiation zone. Mature pericycle cells by the xylem poles in the differentiation zone stimulated to divide further and produce lateral root primordia (Parizot et al., 2007; Fukaki and Tasaka, 2009). Eventually new apical meristem and quiescent center is developed in the lateral roots which promotes the growth of the lateral roots.

2.5.2. Plant growth hormones in root development

2.5.2.1. Auxin

Auxin is the most studied among all growth hormones and plays major roles in all aspects of plant growth and development from embryogenesis to plant tropism (Petrasek and Friml, 2009). Indole 3 Acetic acid (IAA) is regarded as the predominant form of auxin present in plants and play major roles in root development in plants (Ludwig-Müller, 2011). Auxin biosynthesis,

transport, signaling and perception by the plant are the major stages of auxin-mediated root development (Saini et al., 2013).

Auxin biosynthesis is a complex process involving multiple pathways and molecular mechanisms. However the core mechanism of IAA biosynthesis is evolutionarily conserved in many plant species (Saini et al., 2013). IAA can be synthesized through different Tryptophan (Trp) - dependent pathways where Trp plays major roles in synthesis of several precursors of IAA like indole 3 – pyruvic acid (IPA), indole 3 – acetamide (IAM), tryptamine (TAM) etc. which are converted to IAA further. Few evidences suggests that IAA can be synthesized through Trp-Independent pathway although the mechanism is not well understood (Zhao et al., 2009).

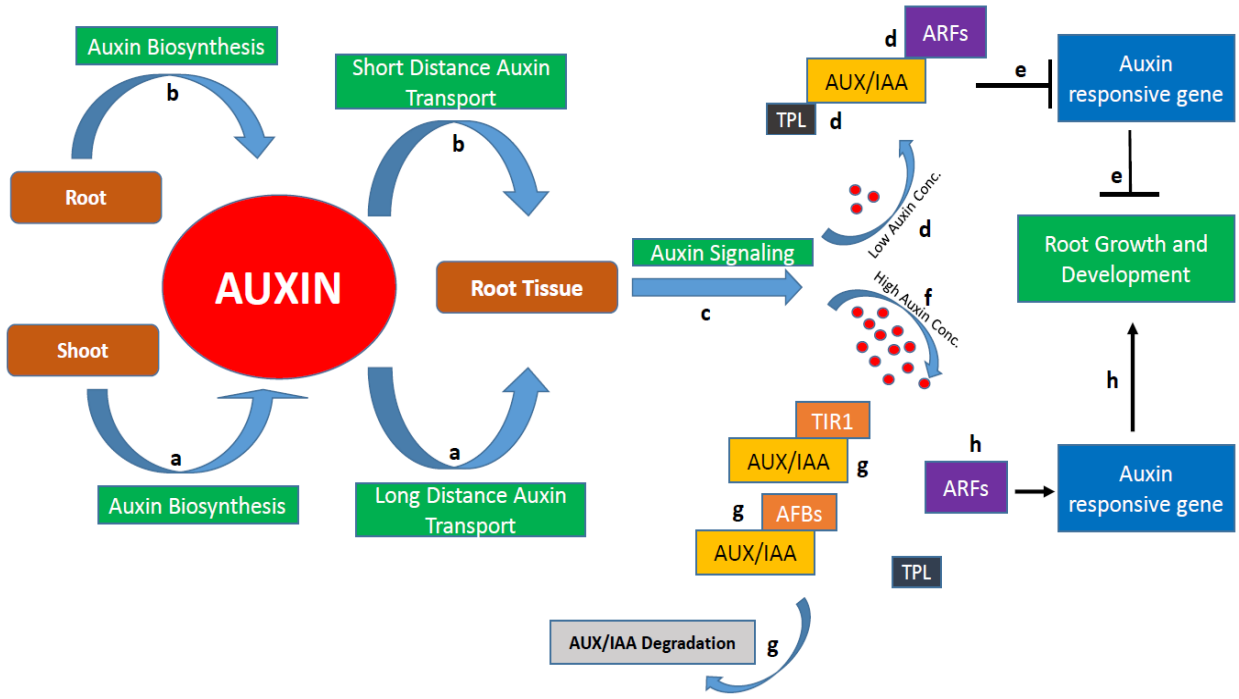

Figure 2.2. Auxin biosynthesis, transport and signaling process. (a) Auxin produced in shoots is transported to the root through phloem with long distance auxin transport system. (b) Auxin produced in roots and transported from cell to cell through efflux and influx auxin careers (c) Auxin signaling. (d) With the low concentration of auxin, AUX/IAA, a transcriptional repressor, binds with AUXIN RESPONSIVE FACTOR (ARFs) with the help of a corepressor TOPLESS (TPL). Some of the ARFs act as transcriptional activator of different auxin responsive genes. (e) Due to binding with repressor (AUX/IAA), ARFs were unable to activate different auxin responsive genes controlling root growth and development. (f) Alternately, when there is high concentration of auxin, (g) AUX/IAA binds with Transport INHIBITOR RESPONSE 2 (TIR2) or Auxin F-BOX PROTEINs (AFBs) and further degraded. (h) ARFs become free to transcriptionally activate auxin responsive genes promote root growth and development.

Auxin is synthesized in both shoot and root tissues (Müller et al., 1998; Ljung et al., 2001; Stepanova, 2005; Overvoorde et al., 2010). Therefore, auxin need to be transported from the area of synthesis to the respective root tissues where it can control the process of root development. As, the auxin transport system can be of two distinct types, long distance transport and short distance transport (Petrasek and Friml, 2009). In long distance auxin transport system, auxin is transported from the shoot to the root tissues through phloem, very similar as the

carbohydrate transport (Goldsmith, 1977; Tsurumi and Wada, 1980). The transported auxin is then added with the auxin synthesized in the root tissue and starts moving to cell to cell, termed as short distance transport (Saini et al., 2013). Unlike the long distance auxin transport, several auxin influx (*AUXIN RESISTANT1 [AUX1]* and *LIKE-AUX1 [LAX1]*) and efflux (*P-GLYCOPROTEIN [PGP]* and *PIN-FORMED [PIN]*) carriers plays important role in cell to cell short distance auxin transport in root (Bennett et al., 1996; Swarup et al., 2001, 2008; Geisler and Murphy, 2006; Petrasek and Friml, 2009). Short distance auxin transport can be of two types, Acropetal auxin transport (from base of the root towards the root tip) and Basipetal auxin transport (from root tip towards the base) (Mitchell and Davies, 1975; Rashotte et al., 2000). Auxin transport in cellular level plays major role in root patterning and differentiation process. Inhibition or disruption of the auxin transport largely affect the root patterning (Aida et al., 2004). Epidermal cells can import auxin through auxin influx carrier *AUX1* to form root hairs and root hair elongation (Jones et al., 2009; Overvoorde et al., 2010). Aida et al., (2004) described the importance of cell auxin level and auxin gradient in expression of *PLT1* and *PLT2* genes which encode AP2 transcription factors responsible for quiescent center speciation and stem cell activities.

Auxin signaling in root tissue depends on the level of interaction between *AUX/IAA* protein family and the *TRANSPORT INHIBITOR RESPONSE 1* (*TIR1*) or *AUXIN F-BOX PROTEIN*s (*AFB*s) (Overvoorde et al., 2010). *AUX/IAA* protein family includes 29 proteins and act as a transcriptional repressor. Most of these *AUX/IAA* proteins have four conserved domains, I, II, III and IV. *AUX/IAA* proteins bind with *TOPLESS* (*TPL*), a corepressor, with domain I (Szemenyei et al., 2008) and *TIR1* with domain II (Tan et al., 2007). On the other hand, they use domain III and IV to interact with other *AUX/IAA* proteins and also to bind with *AUXIN*

RESPONSE FACTORs (*ARF*s) (Kim et al., 1997; Ulmasov et al., 1997). *ARF*s like *ARF5*, *ARF7*, *ARF8*, and *ARF19* are well regarded as the transcription activator of different auxin responsive genes (Liscum and Reed, 2002). *ARF7* and *ARF19* positively regulate the lateral root formation by activating the transcription of *LATERAL ORGAN BOUNDERY DOMAIN* (*LBD*)/*ASYMETRIC LEAVES 2-like* (*ASL*) genes *LBD16/ASL18* and *LBD29/ASL16* (Okushima et al., 2007). With less auxin concentration in root tissue, the *AUX/IAA* protein bind with the *ARF*s with their domain III and IV with the help of corepressor *TPL* and repress the activity of *ARF*s (Szemenyei et al., 2008). With the presence of high concentration of auxin in root tissue, the *AUX/IAA* proteins bind with the *TIR1* or *AFB*s with their domain I and II and soon degrade through Ubiquitin-Proteasome pathway. As a result of *AUX/IAA* and *TIR1* or *AFB*s bond, *ARF*s are now free to activate the auxin responsive genes which play major roles in root the development (Gray et al., 2001; Ramos et al., 2001; Zenser et al., 2001; Mockaitis and Estelle, 2008; Lau et al., 2009).

2.5.2.2. Ethylene (ET)

Involvement of ethylene in root development is mainly through interfering with the auxin biosynthesis and transport process. Auxin plays a positive role in ethylene biosynthesis by upregulating *ACC* (*1-aminocyclopropane-1-carboxylate*) synthase gene which is essential for ethylene biosynthesis (Abel et al., 1995). On the other hand, ethylene controls the expression of two *WEAK ETHYLENE INSENSITIVE* (*WEI2* and *WEI7*) genes which limits the Trp-biosynthesis and negatively affect Trp-dependent auxin biosynthesis process (Woodward and Bartel, 2005). There are evidences of reduced LR formation in *Arabidopsis* with the application of *ACC* which can be reversed by an ethylene antagonist, silver nitrate treatment (Negi et al., 2008). In tomato, ethylene causes significant reduction of auxin content in root tissue (Negi et al., 2009). In addition to the auxin biosynthesis process, ethylene also regulate the polar auxin

transport system (Ruzicka et al., 2007; Negi et al., 2008; Lewis et al., 2011). Ethylene stimulates several auxin efflux (*AUX1*) and influx career (*PIN2*, *3* and *7*) and positively regulate auxin transport from root tip to the elongation zone. As a result, localized auxin accumulation needed for lateral root formation is hampered.

2.5.2.3. Cytokinin (CK)

Cytokinin and auxin cross talk with each other antagonistically to control root development. A member of the auxin repressor *AUX/IAA* protein family, *SHORT HYPOCOTYLE 2 (SHY2)* gene plays the central role in this process. *SHY2* gene is promoted by cytokinin which limit the expression of *PIN* genes (Tian et al., 2003; Dello Ioio et al., 2008). *PIN* proteins are auxin efflux carrier and responsible for optimal auxin distribution to regulate cell division and expansion in root meristem (Billou et al., 2005; Galinha et al., 2007). In contrast, auxin modulate the degradation of *SHY2* which leads to enhance the expression of *PIN* proteins and maintenance of auxin proper gradients required for cell division in the meristematic zone. Experimental evidence suggests gain of function of *shy2* mutant produce smaller root meristem and loss of function of *shy2* mutant produce larger root meristem (Dello Ioio et al., 2008). There is other experimental evidence supporting the negative role of cytokinin in root development. *ARABIDOPSIS HISTIDINE KINASE 2 (AHK2)* and *AHK3* act as a receptor in the cytokinin signaling. Mutant analysis showed that, ahk2ahk3 mutant plants produce larger root system with primary branch and root system due to impaired perception of cytokinin (Riefler, 2006).

2.5.2.4. Gibberellin (GA)

Gibberellin plays several roles in controlling root growth in plants. One of the mostly studied GA regulations in root control is GA mediated degradation of growth repressor DELLA

proteins in association with auxin. DELLA proteins such as *GAI* (*GA insensitive*) and *RGA* (*repressor of GA1-3*) usually repress the GA signaling and GA regulated root growth and development (Dill and Sun, 2001; Saini et al., 2013). With the increased level of auxin, GA biosynthetic gene *GA2O3* produce increased amount GA which bind with GA receptor protein *GID1* (*GA insensitive dwarf 1*) protein (Tanimoto, 2005; Saini et al., 2013). This *GA-GID1* binding activates the GA signaling which enhances the degradation of DELLA proteins and reduce their growth inhibiting effects on roots (Dill and Sun, 2001; Tanimoto, 2005; Saini et al., 2013). Ubeda-Tomás et al., (2009) treated wild type *Arabidopsis* seeding with a GA biosynthesis inhibitor PAC (Paclobutrazol) which inhibited the root growth. They found that size of the root meristem and length of the mature cells were significantly reduced in the PAC treated seedlings. They also reported that cell production rate was lower in the GA biosynthesis mutant *Arabidopsis* seedling.

In addition, several studies reported the inhibitory effect of GA on lateral root growth. Berova and Zlatev, (2000) reported that GA synthesis mutant tomato plants produce higher number of lateral roots. Similarly, GA deficient and GA insensitive *Populus* root produce lower number of lateral roots than their wild types (Busov et al., 2006). Exogenous application of GA on GA deficient mutant *Populas* inhibited lateral root formation (Busov et al., 2006; Gou et al., 2010). GA also plays an important role in root thickening too. Inhibition of GA biosynthesis results reduced root growth but induce the expansion of the cortex cells resulting thickening of the root system in the elongation zone (Tanimoto, 2005). These can completely alter by the external application of GA.

2.5.2.5. Brassinosteroid (BR)

BR plays variety of roles in root growth and development including root cell elongation, root length, root meristem size, lateral root and root hair formation, and gravitropism (Wei and Li, 2016). Hacham et al., (2011) showed that *BRI1* activity in the epidermis regulates the root meristem size in *Arabidopsis*. Mutant *bri1* plants exhibited reduced root meristem which resulted impaired cell cycle activity and cell expansion. BR regulates the root meristem size in a concentration dependent manner (Gonzalez-Garcia et al., 2011; Hacham et al., 2011). Both loss of function and gain of function BR mutant plants produced shorter sized root meristem indicating an optimum BR signaling is required for proper root meristem growth in *Arabidopsis* (Gonzalez-Garcia et al., 2011). Bao, (2004) showed that Mutant *bir1 Arabidopsis* plants showed reduced number of lateral roots which can be recovered by external application of BR. BR, through facilitating acropetal auxin transport promotes initiation of lateral root primordia rather than promoting growth of lateral roots in later developmental stage (Bao, 2004; Wei and Li, 2016).

2.5.2.6. Abscisic acid (ABA)

ABA is well regarded for its role in shoot and root growth during different abiotic stresses and help plants to adapt through morphological and physiological modification (Chen et al., 2006). Early studies showed that with endogenously increased level of ABA reduced shoot growth but maintain root elongation in maize under water deficit condition (Saab et al., 1990). However, the mechanism was unknown at that time. Several studies later showed that under low water potential increased concentration of endogenously accumulated ABA actually inhibited the ethylene biosynthesis to maintain continuous root elongation (Spollen et al., 2000; Sharp and LeNoble, 2002). Later interaction of ABA and auxin (*Aux/IAA*) were detected in the process.

With high ABA concentration, ethylene production was restricted as well as transportation of *Aux/IAA* to the root tip was inhibited which resulted enhanced root growth (McAdam et al., 2016). In contrast, Xu et al., (2013) showed that under moderate water stress condition accumulation of ABA increased auxin transport to the root tip and increase proton secretion resulting primary root elongation and root hair formation in rice and *Arabidopsis*. ABA plays major role in regulating lateral root formation too. Two transcription factor *ABA insensitive 3* and *4* (*ABI3* and *ABI4*) encodes two different binding domain and showed contrasting effect on lateral root growth (Saini et al., 2013). *ABI3* has conserved B3 binding domain which interacts with *Aux/IAA* and inhibit lateral root formation (Brady et al., 2003). On the other hand, *ABI4* encodes AP2 domain which promotes lateral root formation. However, overexpression of *ABI4* caused inhibition of auxin efflux carrier *PIN1* which can restrict lateral root growth in *Arabidopsis* (Shkolnik-Inbar and Bar-Zvi, 2010).

2.5.2.7. Polyamines (PA)

Polyamines are low molecular weight polymers associated with different developmental stages including cell proliferation, active growth and metabolism (Kusano et al., 2007; Saini et al., 2013). Several studies reported role of polyamine in root growth and development. Hummel et al., (2002) reported that PA biosynthesis inhibitors reduced the endogenous PA content and root growth was significantly inhibited in a sub-Antarctic cruciferous species *Pringlea antiscorbutica* at low temperature. Rooting during in vitro growth of sweet orange was significantly improved when exogenous PA were applied in the growing media (Mendes et al., 2011). This process was reversed by applying PA biosynthesis inhibitor in the growing media. Martínez Pastur et al., (2007) also reported that polyamine improved in vitro rhizogenesis in species *Nothofagus nervosa.* It has been reported by few studies that PA positively affected

adventitious root growth in vitro i.e. poplar shoots (Hausman et al., 1994), apple rootstock (Naija et al., 2009) etc.

2.5.2.8. Jasmonic acid (JA)

Jasmonic acid and its derivatives are lipid derived compounds and regarded as plant hormone (Corti Monzón et al., 2012). They are mainly characterized as growth regulators associated with plant defense response against insect wound and for certain bacteria and fungi (Farmer et al., 2003; Wasternack, 2007). Besides defense responses, JA are associated with different aspects of growth and development in plants. There are several reports of JA mediated root growth inhibition in different plant species *Arabidopsis* (Staswick et al., 1992), *Oryza sativa* (Wang et al., 2002), *Allium cepa* and *Phaseolus coccineus* (Maksymiec and Krupa, 2007). However, in another study, Gutierrez et al., (2012) showed that Auxin regulates adventitious root growth in *Arabidopsis* through JA homeostasis. Exogenous application of JA and inhibitor of JA reduced primary root growth and lateral root growth in *Helianthus annuus* in an auxin independent manner (Corti Monzón et al., 2012). However, some other studies also reported JA mediated restriction of auxin transport genes *PIN1* and *PIN2* in *Arabidopsis* resulting auxin accumulation in root basal meristem indicating crosstalk between JA and Auxin in root growth (Sun et al., 2009).

CHAPTER 3: A COMPARATIVE STUDY ON ROOT TRAITS OF SPRING AND WINTER CANOLA (*BRASSICA NAPUS* L.*)* UNDER CONTROLLED AND WATER STRESSED CONDITIONS[1]

3.1. Abstract

Root system in canola (*Brassica napus* L.) varies largely in different growth habit types. A study was conducted with five winter and five spring types germplasm to identify the gradual change of root traits at different growth stages under normal and water stressed conditions. Two experiments, controlled condition and water stressed, were conducted in the greenhouse. Data on different root traits were collected at 30, 40, 50 and 60 days after planting. In controlled experiment, no significant difference was observed for root traits between winter and spring types at 30 days after planting. However, significant variations were appeared for taproot length (F= 10.17***) and root dry weight (F = 16.96***) between winter and spring types at 40 days after planting. All other root parameters such as soil level taproot diameter (F= 22.14***), bottom taproot diameter (F= 4.59*), primary root branches (F= 78.70***) and root vigor (F= 47.18***) were significantly higher in the winter types compared to those of the spring types at 60 days after planting. Growth pattern curves indicated that all the root traits of spring types increased in a linear fashion, where the root traits of winter types increased rapidly after 40 days of planting. In water stressed experiment, the water stress was applied from 20 to 60 days after

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planting, and data were taken at 60 days after planting. All the root parameters except taproot length were significantly ($P < 0.001$) decreased in the stressed plants compared to the control plants., where the soil level taproot diameter, bottom taproot diameter, primary root branches, root vigor, and root dry weight were decreased by 43%, 63%, 19%, 31% and 53%, respectively The root growth reduction in stressed winter type genotypes was higher than stressed spring type genotypes. This study indicated that winter type canola generates vigorous root system under normal growing conditions, and ceases root growth the most under water stressed conditions.

Key words: Root traits, water stress, winter and spring *B. napus*.

3.2. Introduction

The root system *provides anchorage* and support to the *plant and allows of* mining water and nutrient from the soil. Deep and vigorous root system can facilitate higher moisture and nutrient acquisition from the soil, which can boost up the yield largely (Marschener, 1998). On the other hand, less vigorous and shallow root system can uptake less amount of moisture and nutrients, which might end up with reduced yield and biomass production. Moreover, shallow root system cannot uptake moisture from deeper soil, and therefore, become vulnerable in drought prone soil. Crop plants use the nutrient and water to perform the necessary metabolic processes, which affect the crop growth and yield positively. For example, maize root system exhibits root growth variation under low phosphorus (P) level in soil and the genotypes having higher lateral root growth were able to uptake more P and maintained good crop stand (Zhu and Lynch, 2004). Seed yield is positively correlated with longer root system in Rice (Steele et al., 2013), canola (Rahman and McClean, 2013), soybean (Brown and Scott, 1984) and maize (Hochholdinger et al., 2008).

Three canola growth habits, winter, semi-winter, and spring types differs greatly in terms of shoot morphology, root growth and flowering time. Winter types canola have vigorous root system with higher root length, root diameter, root mass and root branches than the spring types canola (Rahman and McClean, 2013; Arifuzzaman et al., 2016). With these superior root characteristics, root system in winter types might be able to cover more area and depth in soil and better access to moisture and nutrient. As the root length of canola is positively correlated with seed yield (Rahman and McClean, 2013), it can be hypothesized that moisture and nutrient uptake capability is higher in winter types canola, which might play a major role in the higher yield capacity over the spring types. These growth habits belong to different genetics groups (Kebede et al., 2010). Therefore, huge variation in the root system of winter and spring canola can serve as significant source of genetic diversity in breeding for high yielding spring canola. Direct selection for root traits in the traditional breeding programs is not very popular due to several constraints. Detecting the root phenotypic variation in a large-scale field trial regarded as one of the main constraints. In addition, root plasticity or preferential growth towards the area of higher moisture and nutrient may deceive plant breeders in highly heterogeneous soil (Arifuzzaman et al., 2016). An alternative, marker assisted selection (MAS) by identifying quantitative trait loci (QTL) associated with the genomic region that control root variation in canola could be a good solution. However, appropriate phenotyping of a trait is always very critical in the process of identifying genomic region associated with that trait.

We observed in a preliminary study that there is no major variation in root traits between winter and spring types at early growth stages, but rather variations are observed in matured plants. There is no report available that monitored the variations of root growth pattern of winter and spring types canola. The lack of adequate information in this regard limits the scope of

proper phenotyping of root traits in a mapping population, as well as traditional selection for root traits in the breeding program. Considering these factors, the objectives of the current study was to detect the plant growth stages at which the variation of root traits initiate and reach to maximum. Our secondary objective was to study the root growth behavior under simulated water stress conditions in spring and winter types canola.

3.3. Materials and methods

3.3.1. Plant materials

A total of 10 canola genotypes, five winter types (Wichita, Lindora-00, KSU 8, KSU 10, Regal) and five spring types (Oro, DH45, Kanada, Regent, Wester), were used for this experiment. The plants were grown in long pots (16"×4") in a greenhouse. A mixture of sand and peat soil in a ratio of 8:2, respectively, were used to grow the plants. The growing media was supplemented with 10g Osmocote® slow-release fertilizer (Scott's Company LLC, Marysville, OH, U.S.A.). Before potting, the pots were lined with plastic bags to facilitate root extraction procedure. The plastic bags were perforated at the bottom to allow the excess *water* to drain out. Plants were watered daily to *saturate* all *pots* and fertilized with water-soluble 20-20-20 fertilizer.

3.3.2. Experimental design

Four sets of experiments with the same germplasm panel (5 winter and 5 spring types in each sets) were grown in the greenhouse. Each set was planted in a randomized complete block design (RCBD) with four replications. Each pot contains one single plant and considered as an experimental unit. These four sets of experiments were grown for four different time periods such as 30 days, 40 days, 50 days, and 60 days after planting.

3.3.3. Data collection

Data were collected from the plants at 30 days after planting (30d), 40 days after planting (40d), 50 days after planting (50d), and 60 days after planting (60d). Data on number of leaves and stem diameter were taken from the freshly harvested plants. The plants were cut at the base of the root. The pots were taken to root washing zone. The roots with plastic bag were taken out from pots, placed on sink containing a fine plastic net, and the soils covering root masses were washed with running water. This procedure facilitates to avoid root loss during washing. Absorbent papers were used to soak the water from the extracted clean root system and kept them for 1 hour at room temperature. Data on taproot diameter were taken at two points, just at the below of soil level where the first root was initiated (soil level taproot diameter) and at 10 cm below from the place of first root diameter (bottom taproot diameter). In addition, data on tap root length, and number of root branches were taken. Total root system were visually scored on the basis of root vigor and root mass on a scale of 1-5 according to Rahman and McClean, (2013), where score 1 represents weak bottom and surface roots, score 2 more bottom and surface roots, score 3 intermediate bottom and surface roots, score 4 strong bottom and surface roots, and score 5 the strongest bottom and surface roots (Figure 3.1). The entire root system of each plant was stored in a perforated plastic bags and dried in 60ºC for 3 days. Data on root dry weight were taken for each plant.

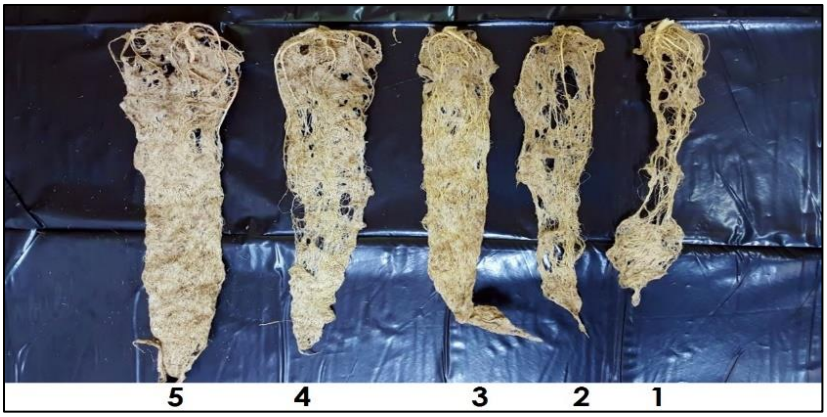

Figure 3.1. Root vigor score from 1-5 (1 represent weak bottom and surface roots, 2 more bottom and surface roots, 3 intermediate bottom and surface roots, 4 strong bottom and surface roots, and 5 the strongest bottom and surface roots).

3.3.4. Data analysis

The root traits of spring types were compared with the winter types within a set. SAS 9.3 statistical software package was used for data analysis. Data from each experimental set (30d, 40d, 50d, and 60d) were analyzed separately to determine if there is any significant difference exists in different root traits between spring and winter types within a set. Line and bar charts with mean and standard deviation for each trait were created by using Microsoft Excel 2013 to explain the progression of the traits throughout the experimental period.

3.3.5. Water stress study

A water stress experiment was conducted simultaneously with the first experiment using the same spring and winter types germplasms to evaluate the response of root growth under simulated water stress conditions. The experiment was set in a randomized complete block design with four replications. Water stress was initiated on plants at 20 days after planting by restraining watering until the plants were wilted. Moisture level across the wilted pots were taken and averaged to identify the moisture level at which the plants were wilted (10% by volume).

The growing media in the pots were allowed to dry at a soil moisture level of 10% by volume followed by water saturation. This water stress was continued until 60 days after planting. The available soil moisture was measured by a soil moisture meter (Spectrum technologies, Inc.). Data were taken from the water stressed plants of 60 days after planting on number of leaves, stem diameter, soil level taproot diameter, bottom taproot diameter, taproot length, number of root branches, root vigor, and root dry weight were taken from each plant using the same procedures described above.

3.4. Result

3.4.1. Stem diameter

At 30 days after planting, winter and spring types plants did not show any significant difference for stem diameter (Table 3.1). However, from 40 days after planting stem diameter was significantly higher ($p > 0.001$) in winter types comparing to those of spring types. Stem diameter in winter types was sharply increasing after 30 days and became stable within 40 and 50 days after planting (Fig 3.2a). In spring types, stem diameter was increasing relatively slow and steady fashion until flowering time at about 40 days after planting.

3.4.2. Soil level taproot diameter

Soil level taproot diameter was not significantly different between winter and spring types at 30, 40 and 50 days after planting (Table 3.1). At 60 days after planting, it was found significantly higher ($P > 0.001$) in winter types compared to the spring types (Table 3.1, Fig 2b). Soil level taproot diameter in both winter and spring types increased with a similar trend until 50 days after planting.

3.4.3. Bottom taproot diameter

Data on bottom taproot diameter were not taken at 30 days after planting. Significant difference ($p > 0.05$) in bottom taproot diameter was observed only at 60 days after planting (Table 3.1). It remained relatively stable up to 50 days after planting and then started to increase sharply in both winter and spring types (Fig 3.2c).

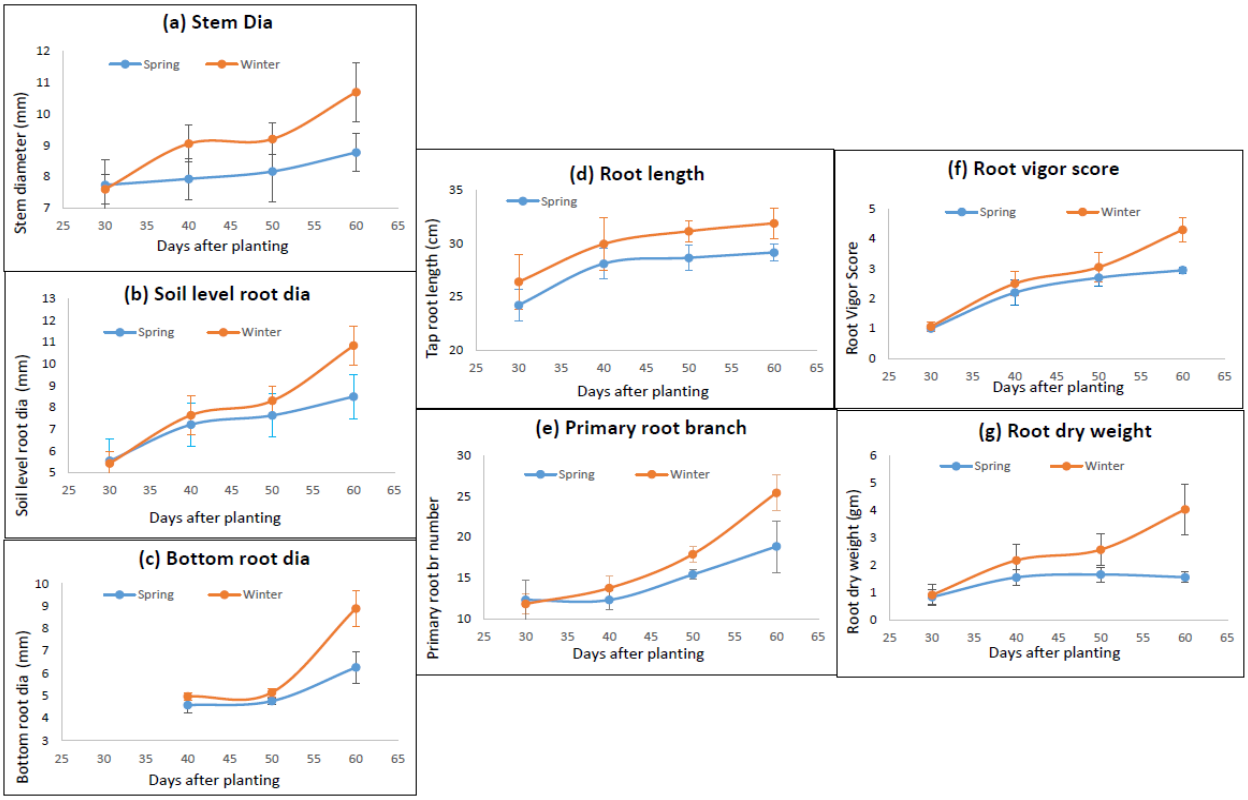

Figure 3.2. Side by side growth trend of different root parameters in winter and spring types from 30 days to 60 days after planting (a= Stem diameter, b= Soil level taproot diameter, c= bottom taproot diameter, d= Tap root length, e=Primary root branch number, f= Root vigor score, g= Root dry weight)

3.4.4. Taproot length

Taproot length was significantly different ($p > 0.001$) in winter types than those of spring types from 40 days after planting (Table 3.1). Both winter and spring types had similar steady growth trend of the taproot length from the very beginning of the experimental period (Fig 3.2d).

3.4.5. Primary root branches

Winter and spring types showed a significant difference ($p > 0.001$) on number of primary root branches at 50 and 60 days after planting (Table 3.1). Number of primary root branches of winter types increased steadily after 30 days of planting where it remained constant until 40 days in spring types followed by a steady increase of root branches (Fig 3.2e).

3.4.6. Root vigor score

Root vigor was found to be higher in winter types comparing to those of the spring types in all four-time periods. However, winter types exhibited significantly ($p>0.001$) higher root vigor at 50 days ($p>0.05$) and 60 days ($p>0.001$) after planting (Table 3.1, Fig 3.3). Root vigor increased with a similar trend in both winter and spring types until 50 days (Fig. 3.2f). A rapid increase after 50 days of planting was observed only in winter types.

Table 3.1. F ratios from analysis of variances for different root traits of winter and spring type canola at different growth stages.

	Source of variance: Type (Winter vs Spring)						
Days after planting	Stem Dia	Soil level taproot dia	Bottom taproot dia	Tap Root length	Primary Root Br. number	Root Vigor score	Root dry weight
30d	0.44	0.04	------	2.64	0.44	1.14	0.04
40d	16.32***	0.44	0.07	10.17***	1.7	2.54	16.96***
50d	12.32***	2.95	0.36	13.77***	9.87***	4.58*	30.32***
60d	23.03***	22.14***	4.59*	14.3***	78.7***	47.18***	73.95***

= significant at 0.05 level; *=significant at 0.001 level*

3.4.7. Root dry weight

Roots dry weight of winter types were significantly higher ($p>0.001$) than the spring types at all stages except 30 days after planting. This trait increased steadily until 50 days in winter types followed by a rapid increase in the next 10 days (Fig 3.2g). On the other hand, root dry weight in spring types increased until 40 days and remain constant after this period.

Figure 3.3. Observed variation between winter and spring type root mass at 50 and 60 days after planting.

3.4.8. Water stress experiment

Water stress was imposed to the plants at the age of 20 days to 60 days after seeding. All the root parameters at 60 days after planting in both winter and spring types were significantly affected under water stressed compared to their normal grown plants (Fig 3.4). The soil level taproot diameter, bottom taproot diameter, root vigor, and root dry weight were significantly ($p>0.001$) reduced in stressed plants of both winter and spring types (Table 3.2). Response to water stressed for root traits reduction of winter type was higher compared to the spring type. For instance, soil level taproot diameter was reduced by 43% in water stressed winter type plants where it is reduced by 32% in water stressed spring type plants (Fig 3.5). Similar response was observed for bottom taproot diameter (63% reduction in winter types, and 53% in spring types), root vigor score (31% reduction in winter types, and 25% in spring types), and root dry weight (53% reduction in winter types, and 32% in spring types).

Table 3.2. F ratios from analysis of variances for different root parameters in winter and spring type canola under control vs water stress condition at 60 days after planting.

Type	Days after planting	Source of variance: Treatment (Drought stress vs control)							
		Stem Dia	Soil level taproot dia	Bottom taproot dia	Tap Root length	Primary Root Br. number	Root Vigor score	Root dry weight	No. of Leaves
Spring	60d	85.1***	43.8***	31.8***	0.4	1.1	45.5***	38.1***	3.8
Winter	60d	87.4***	98.1***	25.2***	3.8	18.7***	138.6***	32.8***	49.2***

***=*significant at 0.001 level*

Number of primary root branches was significantly ($p>0.001$) affected only in the winter types water stressed plants which was reduced by 19% from the normal grown winter types plants (Table 3.2, Fig 3.5). The taproot length was lower in the stressed winter types (5% lower) and stressed spring types (1%) but the rate of reduction was not statistically significant.

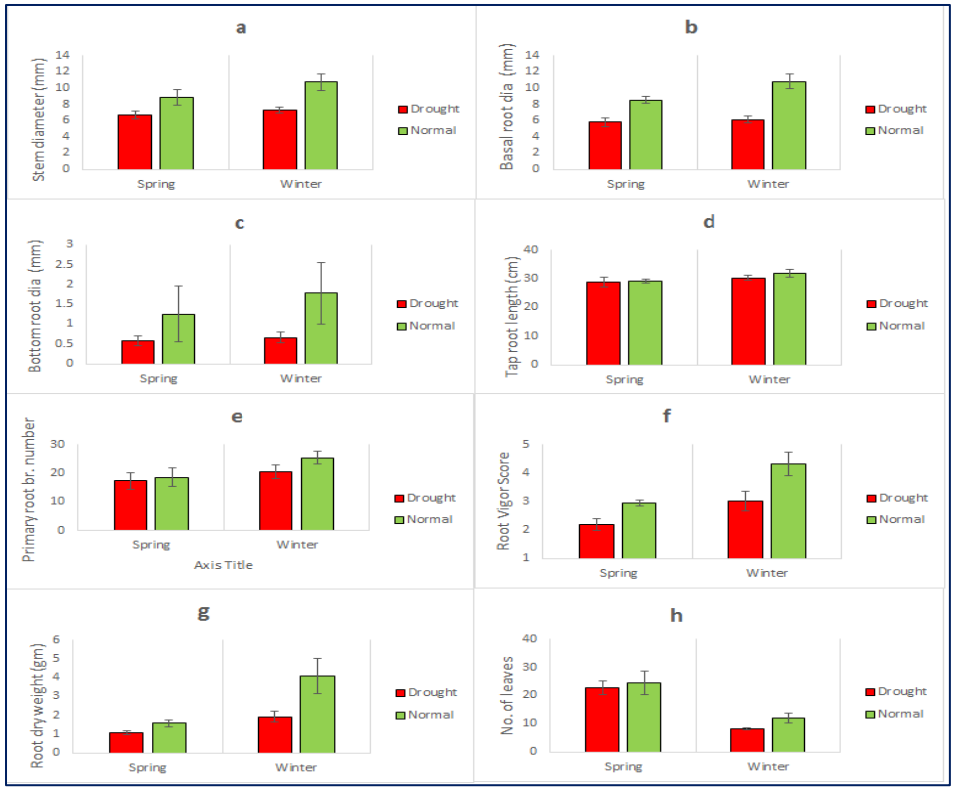

Figure 3.4. Means of different root parameters with standard deviations in spring and winter type canola under water stress and control condition planting (a= Stem diameter, b= Soil level taproot diameter, c= bottom taproot diameter, d= Tap root length, e=Primary root branch number, f= Root vigor score, g= Root dry weight, h= No. of leaves)

Figure 3.5. Variation of root mass under drought stress in winter and spring type canola at 60 days after planting

For the above ground plant parts, stem diameter was significantly ($p>0.001$) affected both in spring and winter types under water stressed conditions (Table 3.2). This trait was reduced by 32% in winter types, and 24% in spring types water stressed plants (Fig 3.5). Number of leaves was reduced significantly ($p>0.001$) by 32% in winter types. In contrast, the effect of water stress on number of leaves in spring types was not statistically significant.

3.5. Discussion

Two canola habits, spring and winter types, are highly distinct in their root morphology. Winter canola produces higher root mass system with higher root length, lateral root brunches and thicker taproot compared to those of spring canola (Rahman and McClean, 2013; Arifuzzaman et al., 2016). Kebede et al., (2010) reported that the winter types are belong to a very distinct genetic group than the spring canola and can be used as a source of genetic diversity to improve spring canola. Along with other traits, superior root traits of winter canola can be introgressed into spring canola to increase the seed yield. Rahman and McClean, (2013) reported that there is a huge difference of root vigor between winter and spring types canola at flowering stage. However, no report yet available to indicate the growth stage at which the root traits variation occurred and maximized between winter and spring types. Therefore, we have conducted the current study to identify the growth pattern of different root traits in four different time points in both winter and spring types canola. We also conducted a water stressed study to investigate the root growth pattern under stressed conditions.

We did not find any significant differences on root traits between winter and spring types at 30 days after planting. This observation is consistent with our small-scale growth pouch study (unpublished data) in which there was no significant variation of root traits between winter and spring types at early growth stage. Wells and Eissenstat, (2002) reported that the root and leaf

formation occur simultaneously in plant until the reproductive stage. Therefore, there is not much difference on root traits at early growth stage of plant. The spring type canola germplasm usually flowers at 40 days after planting in the greenhouse. It is assumed that the plants share more energy to reproductive stage for bud initiation and pod formation. Therefore, roots and vegetative growths are stopped or significantly reduced at reproductive stage. Ledent et al., (1990) reported that leaf initiation is ceased during the tassel formation in maize. Winter canola does not flower without vernalization. At 50 days after planting, the number of primary root branches and root vigor were significantly higher in winter types compared to the spring types. At 60 days after planting, all the root parameters showed significant difference between these two types. The reason of this difference is due to the root and vegetative growth of winter canola plants continue at and after 40 days of planting when the spring types start to initiate flower and stop or reduced the root and vegetative growth. Flowering time changes the energy from vegetative growth to reproductive growth that influences the overall fitness of plant (Michaels, 2009; Posé et al., 2012). This changes of fitness are correlated with many traits including vegetative biomass (Jiaqin et al., 2009; Edwards et al., 2012) and a variety of root traits (Bolaños and Edmeades, 1993; Mitchell-Olds, 1996; Lou et al., 2007). Cheng et al., (1990) reported that the rate of root growth is higher at early plant developmental stages which greatly decreased during reproductive growth stage. This above ground vegetative growth of winter canola might be an indication of the nature of vigorous root growth at below ground.

We have developed a growth pattern curve for all root traits to understand the variations between winter and spring types. Stem diameter, root dry weight, primary root branches, root vigor, and dry root weight in spring types were grown relatively in a steady fashion, where the winter types had a rapid increase from 40d to 60d after planting. This observation indicated that

the root trait variations between winter and spring types initiated at around 40 days after planting. Rahman and McClean, (2013) observed a significant difference of root vigor of winter and spring types during flowering time of spring plants.

With the increasing popularity of molecular breeding methods, high throughput genotyping obtained a substantial improvement in last two decades, however, phenotyping did not receive much attention yet (Zhu et al., 2008). For the complex traits like roots, phenotyping for large scale association study is even more difficult. The pattern of root growth variation observed in this study will be very useful in the future study of large-scale association mapping for different root traits in canola. Phenotyping at 50-60 days after planting will be most effective time to capture maximum amount of phenotypic variation for different root traits in canola. This information might also be helpful in selecting or phenotyping individual root trait for special need.

Crop growth and yield are significantly affected by drought stress (Martin et al., 2006; Saidi et al., 2010). Crop root system plays a vital role in avoidance or adapting plants under low moisture content in the soil (Loomis and Connor, 1992). Modification or alteration of root system under different abiotic stress including drought is a common adaptive measure of plants. In this process, different root traits comprising the whole root system, may respond differently under low water regime (Franco et al., 2011; Licht et al., 2013). We have investigated the behavior of different root traits in both spring and winter types under simulated water stress conditions. Watering was restrained at 20 days after planting until the plants wilted (moisture level 10% by volume) and then saturated with water. This cycle continued until 60 days after planting. Soil level taproot diameter and bottom taproot diameter were found significantly higher ($p > 0.001$) in both spring and winter types of control plants comparing to those of stressed

plants. This finding is consistent with those observed in decreasing root diameter under low water conditions in pea (Eavis, 1972), soybean(Read and Bartlett, 1972), maize (Sharp et al., 1988; Liang et al., 1997) and Silene vulgaris (Franco et al., 2008). Sharp et al. (1988) concluded that, decreasing root diameter is an adaptive measure under low moisture regime so that plant concentrate their resources for root elongation to reach water level. A significant reduction was observed in root vigor and root dry weight of winter and spring types grown in water stressed conditions compared to their normal growing conditions. Again, this is a common response of water stressed plants (Martin et al., 2006; Saidi et al., 2010). In *Arabidopsis*, reduction of root dry weight under severe water stress has been identified (van der Weele et al., 2000).

We did not find any significant difference in root length between winter and spring types under controlled and water stressed conditions. This result is in agreement with Licht et al., (2013) who reported that soybean root elongation was unaffected under water deficit condition. Saidi et al. (2010) observed similar phenomena in maize where no significant difference for total root length was found between different water potential in soil. Franco et al. (2008) studied root and shoot growth in *Arabidopsis* at very early stage in nutrient-agar media and reported that the root elongation was actually stimulated under a certain limit of water potential deficit. Plants usually improve osmotic adjustment at the root growing zone under low moisture availability which might help plants to maintain their root elongation (Martin et al., 2006).

Upper ground traits such as stem diameter and number of leaves were significantly decreased ($p>0.001$) in water stressed plants compared to control plants of spring and winter types. To our knowledge, there is no report available on the effect of water stress on stem diameter and leaf number. These changes are expected as many researchers reported that the

effect of water stress on shoot growth is higher than the root growth (van der Weele et al., 2000; Franco et al., 2008; Saidi et al., 2010; Licht et al., 2013).

3.6. Conclusion

We have observed a differential root growth of spring and winter types canola starting from 40 days after planting. The root growth of spring types significantly reduced at 40 days after planting when the plants start to initiate buds. The winter types do not flower without vernalization and therefore it continues to grow for roots and shoots. All the root traits of the winter type cultivars are highly affected under water stressed conditions over the control experiment. However, this affect is much lower in stressed spring types cultivars compared to their control study. This might be due higher water requirement by the winter type cultivars as they possess higher root and shoot vigor compared to the spring types. In addition, it could be a fact that spring types had higher relative water use efficiency compared to the winter types. However, future investigation is needed to confirm this hypothesis.

CHAPTER 4: GENOME WIDE ASSOCIATION MAPPING AND CANDIDATE GENE MINING FOR ROOT ARCHITECTURAL TRAITS IN RAPESEED/CANOLA (*BRASSICA NAPUS* L.)[2]

4.1. Abstract

Rapeseed/canola (*Brassica napus* L.) root system varied widely among the winter and spring growth habits in later growth stages. In this study, we have phenotyped seven different root architectural traits with a diversity panel consisting of 224 *B. napus* accessions grown in greenhouse during 2015 and 2016. A genome-wide association study (GWAS) with 37,500 single nucleotide polymorphism markers was conducted to detect marker trait association. A total of 52 significant marker loci were identified at 0.01 percentile tail *P*-value cutoff for different root traits, ten loci for root length (RL), eleven loci for root angle (RA), nine loci each for number of primary root branches (PRB) and root dry weight (RDW), seven loci for root vigor score (RVS), and six loci for two root diameter (R_1Dia and R_2Dia). Majority of those significant marker loci were distributed on five chromosomes, A01, A02, A04, C03 and C06. Twenty-two candidate genes related to root traits and root development were detected within 50 kbp upstream and downstream of different significant markers. Three of these candidate genes, *P-glycoprotein 6* (*PGP6*), *Tetraspanin 7* (*TET7*) and *ARABIDILLO-2* were detected within the marker loci chrC03_12098594 (RL), chrA01_8813067 (PRB), and chrA04_rand_54410 (R_1Dia). Multiple marker loci associated with different root traits were detected within a close physical distances

[2] This chapter is co-authored by Muhammad Arifuzzaman and Mukhlesur Rahman. MA and MR formulated the experiment. MA conducted the experiment, collected the data, analyzed the data and wrote the manuscript. MR helped in analyzing the data and reviewed the manuscript.

on chromosome A01, A02, A04 and C03 indication possible co-localization of the loci for different root traits. Twelve significant markers were validated for the marker-trait association of PRB, RVS, RL and RA in 20 germplasm accessions. This is the first report on understanding the molecular basis of the natural variation in root system architecture between winter and spring *B. napus* growth habits at a later growth stages.

Key words: GWAS, root traits, *Brassica napus*

4.2. Introduction

*Brassica napu*s (AACC; 2n=38) is an amphidiploid species formed by ancient natural hybridization between two diploid species *B. rapa* (AA; 2n=20) and *B. oleracea* (CC; 2n=18) (U 1935). It has a relatively short domestication history of only 400-500 years (Gómez-Campo and Prakash, 1999). "Canola" or "***C*an**adian ***O***il ***L***ow ***A***cid" was developed by lowering the erucic acid and glucosinolate content of *B. napus* in 1970's and is regarded as one the most phenomenal achievement in the field of traditional plant breeding. Within five decades, canola become world second most important edible oilseed crop after soybean (Huang et al., 2016). Global production of canola oil reached nearly 27 million metric tons during 2014-2015 (Elahi et al., 2016).

Canola is cultivated worldwide in the form of three growth habits, spring canola (mainly grown in Canada, Australia and parts of USA), winter canola (mainly grown in Europe) and semi winter types (mainly grown in China). These growth habits represent different genetically and morphologically diverse groups (Kebede et al., 2010). This genetic diversity between the growth habits can be utilized to improve the genetics of both spring and winter canola (Rahman and Kebede, 2012). Winter canola is superior in terms of yield comparing to the spring canola and

possess vigorous plant and root characteristics (Rahman and McClean, 2013; Arifuzzaman and Rahman, 2017).

Root system in plants plays the major role in water and nutrient uptake from the soil and transport it to other plant parts. It also provides storage to the acquired water and nutrients and anchorage to the aboveground plant (Saini et al., 2013). Therefore, size of the root mass, root organs and their distribution in the soil can impact plants ability to uptake water and nutrients from the soil. The roots with primary root branch number, lateral branch number, length, diameter and angle of different root components and their spatial arrangement in the soil is referred as the "root system architecture" (Lynch, 2007; Koevoets et al., 2016). Different components of root system architecture were shown to have direct correlation with the yield in various crops. Few examples are, root length in rice (Steele et al., 2013), root hair in maize (Hochholdinger et al., 2008), root vigor in canola (Rahman and McClean, 2013). In addition, plants' ability to cope with different abiotic stresses are largely depended on the plant root system architecture as roots are the first organs that sense and respond to the water and nutrient deficit or excessiveness in the soil (Lynch, 2007, 2011; Hochholdinger, 2016).

Breeding efforts in field crops throughout the last century were mostly focused on improving aboveground phenotypic traits (Koevoets et al., 2016). Despite of having such important roles in plant growth, development and production, below ground root system remains as the least utilized traits for crop improvement (Herder et al., 2010; Wachsman et al., 2015). One of the main constraint regarding direct selection of root traits in the field is labor intensiveness. Phenotyping root traits involves digging, breaking soil crust and washing which discourage breeders to emphasize on large scale selection effort for superior root characteristics. Another difficulty is the plasticity of the root growth. Nutrients and water is not evenly

distributed in the soil. Plant root system can sense the availability of water and nutrients in the soil and tends to grow towards that direction (Malamy, 2005; Smith and De Smet, 2012).

With the advent of high throughput phenotyping and genotyping techniques, identifying quantitative trait locus (QTL) and marker assisted selection for complex traits become more popular now a days. QTL associated with different root traits were discovered in different crops. Several QTL associated with root length and thickness in rice were identified (Steele et al., 2013) and incorporated those in an *Oryza indica* variety through MAS. There are few other example of identification and successful introgression of root associated QTL in different crops, i.e. wheat (Placido et al., 2013), chickpea (Varshney et al., 2013) etc. However, identification of QTL associated with different root traits in *Brassica* species were conducted mostly in response to nutrient deficiency or drought condition (Arifuzzaman et al., 2016). Several QTL were identified for, root vigor (Arifuzzaman et al., 2016), root dry weight and primary root number under boron deficiency (Shi et al., 2011), root mass under drought condition (Fletcher et al., 2015) in *B. napus* and taproot characteristics in *B. rapa* (Lu et al., 2008)

Genome wide association mapping is now a very popular and widely used to identify markers and QTL associated with a particular trait (Lakew et al., 2013). It takes into account several ancestral recombination events during detecting the association between trait and markers in a diverse germplasm panel, whereas, in traditional QTL mapping, only a very few recombination events are taken into consideration between two parents (Gómez et al., 2011). The higher the diversity of the germplasm panel, the higher the historical recombination events are taken into consideration during association mapping approach which ultimately yield higher resolution molecular map (Rahman et al., 2016). A high density molecular markers panel covering whole genome of the species is required for a successful association mapping (Begum

et al., 2015). Single nucleotide polymorphism (SNP) markers are currently most popular choice due to their abundance in the genome, user friendly nature and low cost development (Zhu et al., 2008; Chitwood et al., 2016). The objective of the current study was to identify significant markers and candidate genes associated with the different root architectural traits in greenhouse.

4.3. Materials and methods

4.3.1. Plant materials and experimental design

A total of 224 genotypes were planted in a greenhouse of North Dakota State University, Fargo, ND, USA during 2015 (E1) and 2016 (E2). The accessions were consisting of three growth habits, spring, winter and semi-winter types of rapeseed/canola, and have diversified sources of origin (Appendix Table A1). They were planted in a randomized complete block design (RCBD) with three replications. Longer pots (40 × 10 cm) were used to allow roots to grow deeper. Each pot contains a single canola plant and considered as an experimental unit. Sand and peat soil were mixed together at 8:2 ratio and used as potting mix. The growing media was supplemented with 10g/pot Osmocote® slow-release fertilizer (Scott's Company LLC, Marysville, OH, USA). Transparent plastic bags (perforated at bottom) was lined up with each pot before planting. Plants were watered twice daily and fertilized with water-soluble 20-20-20 fertilizer once a week. Temperature of the greenhouse was maintained as 25 C with 16 hours of photoperiod. Natural sunlight was supplemented with 400 W HPS PL 2000 lights (P.L. Light Systems Inc., Beamsville, Ontario, Canada).

4.3.2. Phenotyping

Roots were extracted from fully grown 55 days old plants. Plant canopies were cut at one inch above of the soil level. The pots with the root system were transferred to the root washing

zone in the greenhouse. Plastic bags were pulled out from the pot keeping the root system intact inside the plastic bag. The whole root system with plastic bag were placed on a perforated base and the plastic bag was cut and removed. Root system was washed carefully with running water while kept on the perforated base. This allowed water and soil media pass through the perforated base and leaving the washed root system on the base. Excess water from the washed root samples were soaked out by using absorbent papers and kept them for one hour in room temperature before data taking (Fig. 4.1). This method is an adaptation of "Mesocosms for Root Evaluation" procedure developed by Roots lab, University of Pennsylvania for maize root evaluation.

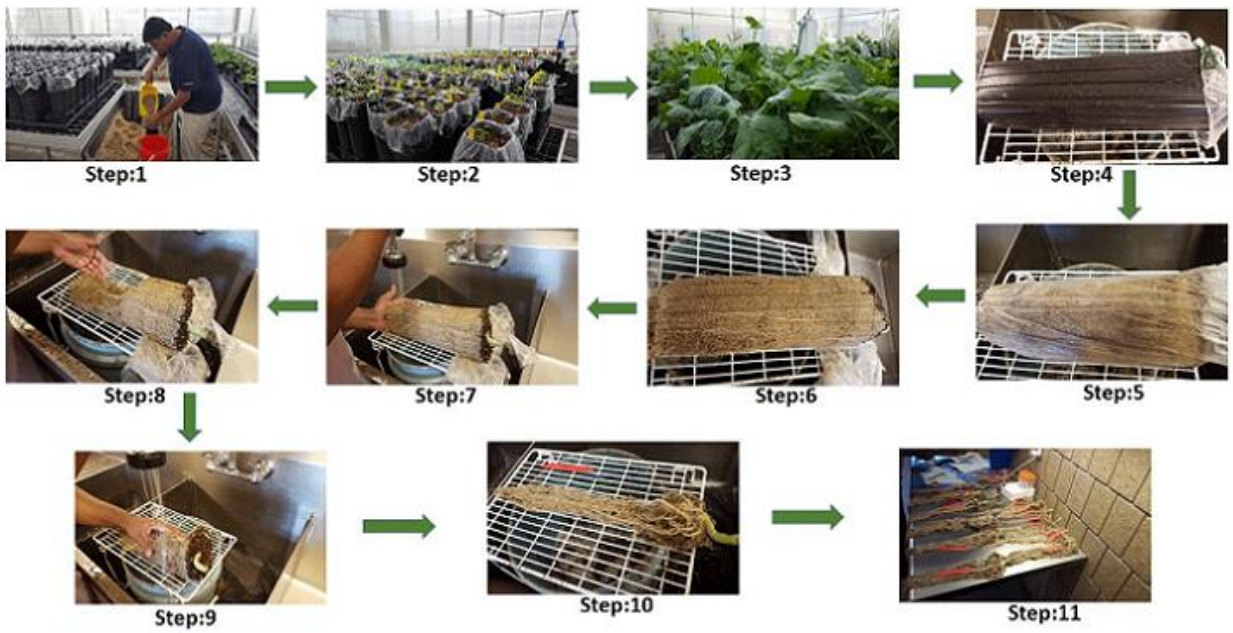

Figure 4.1. Root phenotyping procedure in the greenhouse at a glance.

Data on taproot diameter from 224 genotypes were taken at two points, at soil level where the first root was initiated (R_1Dia) and at 10 cm below from the place of first root diameter (R_2Dia) (Appendix Table A1). In addition, data on tap root length (RL), root angle (RA) and

number of primary root branches from taproot (PRB) were taken from 198 germplasm accessions (Appendix Table A1). Total root system of these 198 germplasm accessions were visually scored on the basis of root vigor and root mass on a scale of 1-5 (RVS) according to (Arifuzzaman and Rahman, 2017), where score 1: weak bottom and surface roots, score 2: more bottom and surface roots, score 3: intermediate bottom and surface roots, score 4: strong bottom and surface roots, and score 5: the strongest bottom and surface roots. The entire root system of each plant was stored in a perforated plastic bags and dried in 60 ºC until constant weight. Data on root dry weight (RDW) were recorded from 177 germplasm accessions (Appendix Table A1). Data on all the traits were taken in both years except root diameters (R_1Dia and R_2Dia) which were measured only in 2016.

4.3.3. Statistical analysis

Data were analyzed in SAS 9.3 (SAS Institute Inc., USA) for individual years 2015 and 2016 denoted by E1 and E2, respectively, in this study. Medians were calculated only for the non-parametric dataset, root score (1-5), in order to follow non-parametric methods to construct two-way analysis of variance (ANOVA). In all other cases means were used to create two-way ANOVA. Data from two years were combined if the ratio of the effective error variance for each trait is less than 10 fold (Tabachnick and Fidel, 2001; Elias and Manthey, 2016) and denoted by "Comb" throughout the current manuscript. Spearman correlation coefficient (only for root vigor score) and Pearson's correlation coefficient among all traits and were calculated.

4.3.4. Genotyping

The genotypes used in this experiment are a subset of the 366 core *B. napus* germplasm collection, originally genotyped for diversity study and SNP detection (Michalack et al.

unpublished). DNA was extracted from vacuum dried young leaf tissue by using Qiagene DNeasy kit Qiagen, CA, US) following the manufacturers protocol. Samples were optimized and sent to Institute of Genomic Diversity (IGD), Cornell University for Genotyping by Sequencing (GBS). GBS libraries were prepared following the protocol described by Elshire et al., (2011), which utilizes single cutter *ApeKI* enzyme for digestion. Each libraries were barcoded and sequenced by Illumina GAII sequencer. 100 bp single end sequenced GBS data were aligned by using BWA-MEM (Li, 2013). Multi sample SNP calling was performed by using VarScan (Koboldt et al., 2012b). Identified SNPs were imputed for missing allele by using FastPHASE (Scheet and Stephens, 2006) and finally 42,575 SNPs were obtained. For the current experiment, SNPs with less than 5% minor allele frequency (MAF) were removed for further analysis. The name of each SNP marker consists of chromosome number and physical position of that marker. For example, a SNP marker located on 123456 bp of chromosome C04 was named as "C04_123456".

4.3.5. Marker-trait association

Association analysis were performed in Tassel 5 using LS mean data for each year separately and by combining data across both years. Principle components (PC) were calculated to account for population structure to prevent falls marker-trait association. Number of principle components that explains 25% and 50% variation in the population were used as two separate regression models. In addition, an identity by state (IBS) kinship matrix was estimated to account the relatedness of the populations. Finally, six regression models were developed, 1. Naïve (without accounting population structure and relatedness), 2. PC controls for 25% variation in population structure, 3. PC controls for 50% variation in population structure, 4. Kinship (accounts for relatedness in the population), 5. PC (25%) + Kinship (accounts for both variation

in population structure and relatedness), 6. PC (50%) + Kinship (accounts for both variation in population structure and relatedness). Among these models, only Kinship, Kinship + PC (25%), and Kinship + PC (50%) are mixed linear models (MLM) and consider both fixed and random effects. All other models are general linear models (GLM) which consider only the fixed effects. All the models were compared on a rank based mean square deviation (MSD) value and best model was selected based on the lowest MSD value (Mamidi et al., 2011). Selected best model for each trait was used for further analysis to detect significant markers for respective traits. Significant markers were called based on the *P*-value of the markers at 0.01 and 0.1 percentile tail of an empirical distribution obtained by 10,000 bootstraps (Mamidi et al., 2014). Manhattan plots were created by using qqman package in R statistical software (Turner, 2014). Log likelihood ratio based R^2 or R^2_{LR} (Sun et al., 2010) was calculated in genAble package in R (Aulchenko et al., 2007) for the most significant markers in the best models to determine the phenotypic variation explained by them.

4.3.6. Candidate gene

B. napus gene models within 50 kbp upstream and downstream of the significant markers were taken into account for candidate gene search. Protein sequences from the gene models were blasted against TAIR 10 protein database to determine the gene annotation. Genes associated with root development were identified based on the gene functions found in previous literatures.

4.3.7. Marker validation

From the significant markers at 0.01 percentile tail, few markers were selected to predict root characteristics in a set of twenty germplasms. Markers were selected based on the

repeatability of the markers across the environment and close physical distance to the candidate genes identified here. The germplasms were planted in the greenhouse in a randomized complete block design with three replication. Different root traits were phenotyped following the similar procedure described above.

4.4. Results

4.4.1. Phenotypic distribution

Phenotypic variations were observed within the germplasm panel for all the traits in both of the years, 2015 (E1) and 2016 (E2), and two years combined (Comb) data. Variation was maximum in E1 comparing to E2, and combined analysis for RL, RA, PRB and RDW (Table 4.1). Coefficient of variance was relatively higher for RDW (E1, E2 and Comb) and for R_2Dia (E2) comparing to other traits. The RL data in E1 and E2, RA data in Comb, and R_1Dia in E2 were normally distributed based on Shapiro Wilk normality test *P*-value (>0.05) (Table 4.1 and Fig. 4.2). Higher family mean basis heritabilty ($h^2 > 0.60$) were observed for PRB (0.58-0.62), RDW (0.89-0.92), RVS (0.81-0.86) and R_2Dia (0.85). For the rest of the traits, low to moderate heritability (h^2 ~0.26-0.49) were observed. Heritability were somewhat constant for each trait between the E1, E2 and Comb.

Table 4.1. Phenotypic variation in different root architectural traits.

Trait	Env.	Germplasm number	Unit	Mean (±std)	Range	CV (%)	Shapiro-Wilk test p value	h^2 (family mean basis)
RL								
	E1	198	cm	23.8 (±3.5)	13.4-34.5	14.8	0.1300	0.30
	E2	198	cm	28.9 (±1.8)	23.3-34.1	6.3	0.0600	0.31
	Comb	198	cm	26.3 (±2.1)	20.2-33.2	7.9	0.0034	0.26
RA								
	E1	198	degree	144.8 (±18.6)	67.6-177.3	12.8	0.0014	0.44
	E2	198	degree	144.2 (±13.2)	87.2-168.3	9.1	0.0001	0.48
	Comb	198	degree	144.5 (±12.7)	107.3-173.6	8.8	0.0160	0.49
PRB								
	E1	198	number	13.5 (±2.9)	8.0-28.0	21.6	0.0001	0.62
	E2	198	number	17.3 (±2.8)	10.3-28.3	16.3	0.0001	0.58
	Comb	198	number	15.4 (±2.4)	10.9-28.1	15.6	0.0001	0.62
RDW								
	E1	177	gm	1.7 (±0.9)	0.4-6.7	51.8	0.0001	0.91
	E2	177	gm	1.6 (±0.6)	0.4-4.6	40.4	0.0001	0.89
	Comb	177	gm	1.7 (±0.7)	0.5-4.8	44.0	0.0001	0.92
RVS								
	E1	198	Scoring	3.1 (±0.9)	1-5	28.8	0.0001	0.86
	E2	198	Scoring	3.4 (±0.8)	1-5	24.8	0.0001	0.81
	Comb	198	Scoring	3.3 (±0.8)	1-5	24.0	0.0001	0.83
R_1Dia								
	E2	222	mm	7.9 (±1.2)	5.4-12.6	13.3	0.0105	0.39
R_2Dia								
	E2	222	mm	1.2 (±0.5)	0.3-3.4	48.7	0.0001	0.85

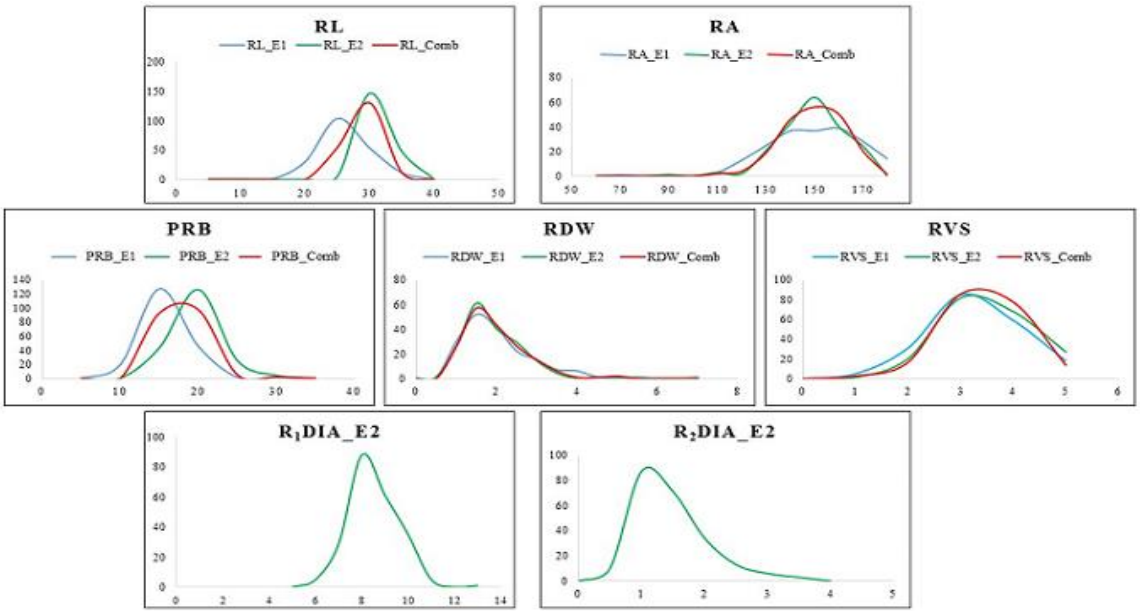

Figure 4.2. Phenotypic distribution of different root architectural traits in E1 (2015), E2 (2016) and combined data set.

4.4.2. Relationships among the traits

Pearson correlation coefficients and spearman correlation coefficients (only for RVS) among the traits within and between the environments were calculated (Fig 4.3). There were moderate but significant (P <0.001) positive correlation between the RL and RDW in E1 (0.25), E2 (0.28) and comb (0.34), and between PRB and RDW in E1 (0.24), E2 (0.37) and comb (0.34). Significant (P <0.001) positive correlation were also detected in RL vs RVS and PRB vs RVS in both environment and when data were combined ranging from (0.33-0.49). Visual root vigor scoring (RVS) was significantly (P <0.0001) correlated with RDW in both environment and combined data set with correlation coefficients ranging from 0.55-0.60. We had root diameters data only for 2016 or E2. R_1Dia moderately correlated with R_2Dia (0.33, P <0.0001). Significant but low to moderate correlation were found in R_1Dia vs all other root parameters (0.32-0.41) except RA and R_2Dia vs all other root parameters (0.27-0.37) except RA.

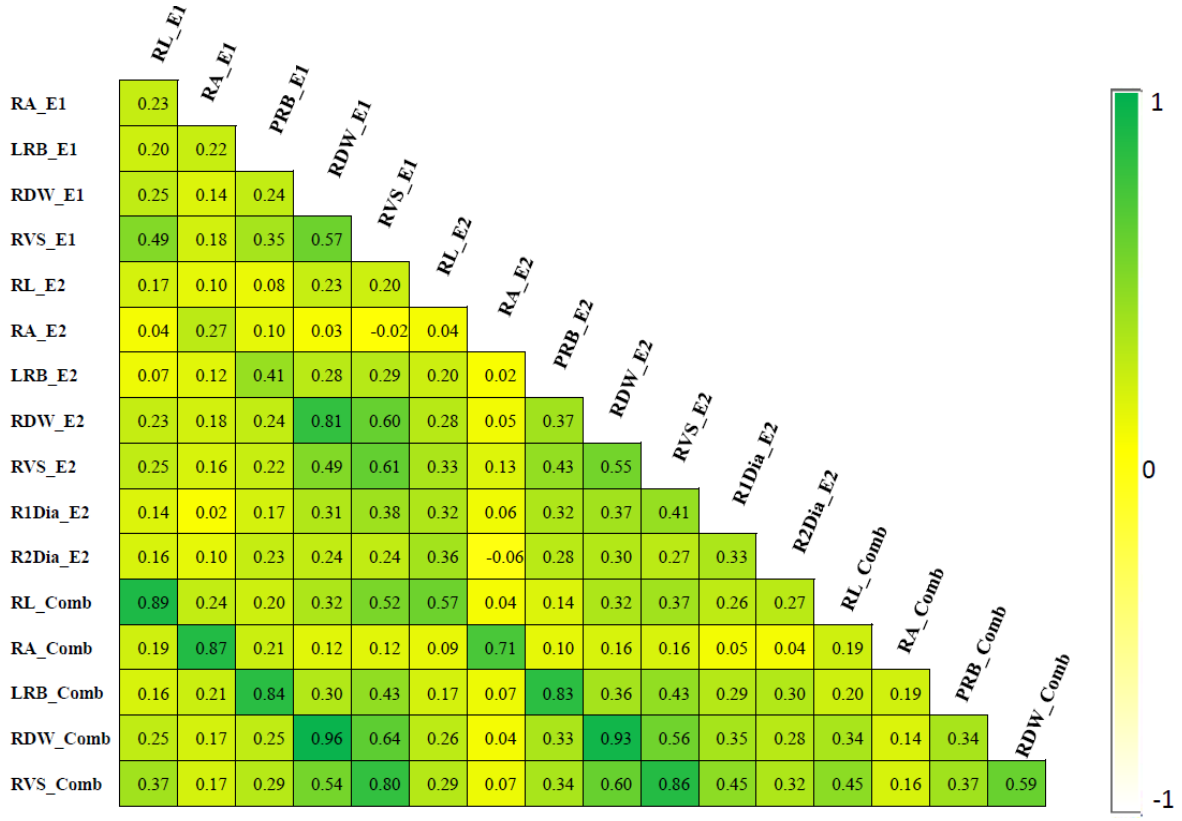

Figure 4.3. Correlation heat map for different root architectural traits in E1 (2015), E2 (2016) and combined data set

4.4.3. Genotypic data and principle component analysis

A total of 42,575 SNPs were derived from the original germplasm panel consists of 366 diverse germplasms. In this study, a subset of 177 (RDW), 198 (RL, RA, PRB, RDW and RVS) and 224 (R_2Dia and R_1Dia) germplasms were used. After correcting the original SNP panel with MAF > 5%, approximately 37,500 markers were retained for different subsets of the germplasms. Population structure were controlled with principle component analysis. Number of PCs accounting for 25% and 50% variation of the population were used to control the population structure. Principal component analysis has grouped the population into three continuous clusters using the first two principal components (Fig 4.4) where PC1 accounts for 13% and PC2

accounts for 9% of the variation. Clustering into three groups were random and could not distinguish between the growth habits or the geographical origin of the accessions.

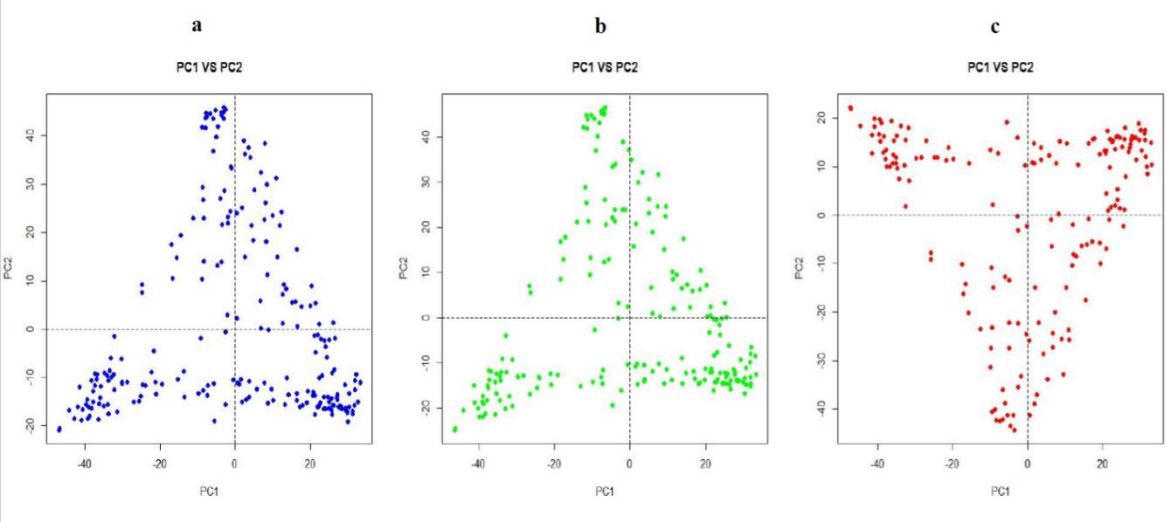

Figure 4.4. Principle component graphs showing the distribution of the populations for first two principle components, (PC1 and PC2) where, PC1 explained 13% of the variation and PC2 explained 9% of the variation in population. (a) 224 genotype for root diameter traits. (b) 198 genotypes for RL, PRB, RA and RVS. (c) 177 genotypes for RDW.

4.4.4. Marker-trait association

We tested six models for every traits for each years individually and for combined data-set to detect the best marker-trait association. Different models were found to be best fitted for different traits in different environment and combined datasets (Table 4.2). Significant markers were identified based on two cut-off criteria, marker *P*-value at 0.01percentile tail of the empirical distribution and at 0.1 percentile tail. SNPs that passed more stringent cut off at 0.01 percentile were the most significant markers for the trait. Significant markers at less stringent cut off at 0.1 percentile tail were taken into consideration only when they were repeated in E1, E2 and combined datasets. Candidate genes were searched for all the significant markers within 50 kbp upstream and downstream of a marker.

4.4.4.1. Root length (RL)

Based on the lowest MSD value, PC4 was selected as the best model for root RL in E1 data set (Table 4.2). Three markers were found significant with 0.01 percentile cut off *P*-value (1.20E-04). Two of these markers' (chrUnn_rand_2975131 and chrAnn_rand_15131528) position in the physical map are unknown based on the *B. napus* reference genome sequence (Chalhoub et al., 2014) whereas, the third marker (chrC06_rand_1272851) was from chromosome C06 and they altogether accounts for 24 % of the total phenotypic variation (Table 4.3). Kinship + PC4, and PC4 were detected as the best model in E2 and combined dataset, respectively. Four markers were found to be significant in each of these data sets at 0.01 percentile *P*-value. The four significant markers in E2 are chrC03_4976549, chrA05_10135011, chrC04_45786858 and chrC03_12098594 (Table 4.3). Altogether they explained 27 % of the total phenotypic variation. Significant markers in combined datasets are on chromosome A10 (13.77 bp), C02 (19.64 Mbp) and C06 (10.54 Mbp). One marker's position is unknown. They altogether accounted for 29.6% of the phenotypic variation. *Brassica* gene model BnaC03g22140D was found within the position of the marker chrC03_12098594 (E2) which shows the best match with *Arabidopsis* gene model AT2G39480 encoding *P-glycoprotein 6 (PGP6, ABCB6)* protein (Table 4.5). Another gene model BnaC03g10280D was found 23.9 kbp downstream of the marker chrC03_4976549 which is similar to *Arabidopsis* gene model AT5G20810 encoding *Small Auxin Upregulated RNA 70 (SAUR70)* protein.

Table 4.2. Best models based on lowest MSD value with cut off P-value at 0.01 and 0.1 percentile tail for each trait in each environment and in combined data set.

Traits	Env.	Best Model	MSD value	*P*-value cut off at 0.01 percentile tail	−log10 (P-value) at 0.01	*P*-value cut off at 0.1 percentile tail	−log10 (P-value) at 0.1
RL							
	E1	PC4	4.53E-06	6.65E-05	4.18	9.69E-04	3.01
	E2	Kin+PC4	9.59E-06	1.14E-04	3.94	9.78E-04	3.01
	Comb	PC4	1.49E-06	1.36E-04	3.87	9.82E-04	3.01
RA							
	E1	PC28+Kin	1.4E-05	6.46E-05	4.19	1.38E-03	2.86
	E2	PC28+Kin	4.37E-06	5.02E-05	4.30	6.48E-04	3.19
	Comb	PC28	2.26E-05	1.03E-04	3.99	9.16E-04	3.04
PRB							
	E1	PC4	3.43E-04	5.47E-05	4.26	1.10E-03	2.96
	E2	Kin	0.000739	2.29E-05	4.64	1.83E-03	2.74
	Comb	Kin	8.38E-05	4.44E-05	4.35	8.10E-04	3.09
RDW							
	E1	PC26	1.27E-03	8.73E-06	5.06	4.69E-04	3.33
	E2	PC26+Kin	7.67E-04	3.29E-05	4.48	1.44E-03	2.84
	Comb	PC26+Kin	1.06E-03	8.12E-05	4.09	9.63E-04	3.02
RVS							
	E1	PC28	7.95E-06	2.47E-04	3.61	1.40E-03	2.85
	E2	Kin+PC28	7.26E-05	1.69E-04	3.77	1.01E-03	3.00
	Comb	PC28	1.07E-05	1.02E-05	4.21	1.24E-03	2.91
R_1Dia							
	E2	Naïve	1.34E-05	1.23E-04	3.91	9.07E-04	3.04
R_2Dia							
	E2	Naïve	3.73E-05	4.47E-05	4.35	1.21E-03	2.92

4.4.4.2. Root angle (RA)

For RA, Kinship + PC28 which accounts for both population structure and relatedness were selected as the best fitted model for both E1 and E2 (Table 4.2). On the other hand, PC28 accounting for only population structure was the best fitted model in combined dataset. At 0.01 percentile, four markers were found to be significantly associated with the RA in each of the

three dataset (Table 4.3). In E1, the four significant markers were from 2 different chromosomes, C03 and C06 and accounts for 24.38% of the phenotypic variation. The four significant markers in E2 were from four different chromosome and explained 24.3% of the phenotypic variation. One significant marker from E2, chrA03_21843293, was also found to be associated with the trait in combined analysis and accounted for 3.0% and 10.9 % of the total phenotypic variation in respective environment. Two candidate genes, *ABC Transporter G28 (ABCG28)* and *Plasma Membrane Intrinsic Protein 2; 4 (PIP2; 4)* were identified from 1.2 kbp and 12.3 kbp upstream, respectively, of the marker chrC03_5436381 (E1) (Table 4.5).

4.4.4.3. Primary root branch (PRB)

The Kinship model accounting for only relatedness was found as the best fitted marker-trait association model for primary root branch in E2 and combined analysis (Table 4.2 2). In E1, PC4, accounting for population structure only, was the best fitted model. Marker chrA02_192565 was the top most significant marker in both E1 and combined analysis (Table 4.3). It accounts for 11.5% of the phenotypic variation in E1, and 8.38% of the phenotypic variation in combined dataset. There were two other markers appeared on both E1 and combined analyses from chromosome A01 (8.81 Mbp) and C03 (1.82 Mbp) at 0.01 percentile tail. In E2, the significant markers at 0.01 percentile were unique and explained 29.4% of the phenotypic variation. A gene model BnaA01g16850D was identified within the position of the marker chrA01_8813067 from E1 and combined dataset (Table 4.5). This gene model matches with *Arabidopsis* gene model AT4G28050 encoding protein *Tetraspanin 7 (TET)* involved in root radial patterning and root morphogenesis. Five other candidate genes were detected within the 50 kbp upstream or downstream from three different PRB associated markers, chrC03_1822934 (*SNRK2, NAC81*), chrA02_1108743 (*PIN8, GASA4*) and chrC04_2352267 (*ARGOS*-like 1).

Table 4.3. Significant markers for different root traits at 0.01 percentile

Trait	Markers	Major allele/Minor allele	Env.	−log10 (*P*-value)	R^2_{LR} (%)
RL					
	chrUnn_rand_2975131	C/A	E1, Comb	5.16	11.69
	chrAnn_rand_15131528	G/A	E1	4.36	9.98
	chrC06_rand_1272851	C/G	E1	4.20	9.62
				Total	24.72
	chrC03_4976549	A/G	E2	4.19	2.34
	chrA05_10135011	A/T	E2	4.06	4.89
	chrC04_45786858	A/G	E2	3.95	13.33
	chrC03_12098594	A/T	E2	3.95	10.09
				Total	27.34
	chrUnn_rand_2975131	C/A	Comb, E1	4.49	10.26
	chrA10_13775810	T/A	Comb	4.31	9.86
	chrC02_19643030	A/G	Comb	4.07	9.34
	chrC06_10542811	C/T	Comb	3.92	9.02
				Total	29.61
RA					
	chrC03_5436381	T/G	E1	4.96	23.30
	chrC03_5436391	C/T	E1	4.93	22.99
	chrC06_33610701	T/G	E1	4.39	1.15
	chrC06_33610722	T/G	E1	4.20	1.24
				Total	24.38
	chrA06_rand_664525	G/T	E2	5.24	4.11
	chrC06_15154006	C/T	E2	5.10	7.20
	chrA09_12827936	G/A	E2	4.65	18.18
	chrA03_21843293	T/C	E2, Comb	4.31	2.99
				Total	24.28
	chrC09_3590337	C/T	Comb	4.69	12.13
	chrA01_12760837	C/G	Comb	4.26	11.08
	chrA03_21843293	T/C	Comb, E2	4.18	10.90
	chrC02_10095030	C/A	Comb	4.13	10.76
				Total	31.15

Table 4.3. Significant markers for different root traits at 0.01 percentile (Continued)

Trait	Markers	Major allele/Minor allele	Env.	−log10 (*P*-value)	R-Sq (%)
PRB					
	chrA02_192565	C/A	E1, Comb	4.90	11.15
	chrA01_8813067	G/T	E1, Comb	4.90	11.14
	chrC03_1822934	G/T	E1, Comb	4.44	10.14
	chrC02_3679554	G/T	E1	4.32	9.89
				Total	27.06
	chrA02_1108743	T/C	E2	5.27	14.00
	chrC04_2352267	T/A	E2	4.80	19.93
	chrC04_2352222	C/T	E2	4.75	19.45
	chrC04_2352228	A/G	E2	4.64	19.09
				Total	29.44
	chrA02_192565	C/A	Comb, E1	5.98	8.38
	chrC01_3445668	A/T	Comb	5.33	19.65
	chrC03_1822934	G/T	Comb, E1	5.08	17.04
	chrA01_8813067	G/T	Comb, E1	4.55	18.79
				Total	39.33
RDW					
	chrC01_11247236	C/T	E1, Comb	6.69	18.78
	chrC06_rand_911028	G/A	E1	6.51	18.35
	chrA07_12603394	C/A	E1, Comb	5.90	16.76
	chrA07_rand_1125045	G/A	E1, Comb	5.51	15.76
				Total	33.11
	chrA02_1172233	A/G	E2	5.24	5.72
	chrA04_4928074	G/A	E2	5.21	0.27
	chrA02_1172218	G/T	E2	5.12	8.01
	chrA01_rand_778885	C/T	E2	4.60	0.08
				Total	8.33
	chrA07_rand_1125045	G/A	Comb, E1	5.34	15.30
	chrC01_11247236	C/T	Comb, E1	5.26	15.10
	chrA07_12603394	C/A	Comb, E1	4.96	14.31
	chrA01_7136448	A/G	Comb	4.51	13.10
				Total	32.55

Table 4.3. Significant markers for different root traits at 0.01 percentile (Continued)

Trait	Markers	Major allele/Minor allele	Env.	−log10 (*P*-value)	R-Sq (%)
RVS					
	chrA04_11064427	A/C	E1, Comb	5.34	18.56
	chrC05_16971678	C/A	E1	4.07	16.34
	chrCnn_rand_27194557	G/A	E1, E2, Comb	3.94	14.42
	chrA01_7949816	A/G	E1, Comb	3.83	13.93
				Total	39.93
	chrCnn_rand_27194557	G/A	E2, E1, Comb	4.11	2.26
	chrC08_37021388	C/T	E2	3.87	1.81
	chrC08_37021410		E2	3.87	1.81
	chrA04_5977698	T/C	E2	3.83	1.48
				Total	5.89
	chrCnn_rand_27194557	G/A	Comb, E1, E2	4.91	12.68
	chrA04_11064427	A/C	Comb, E1	4.34	11.29
	chrA01_7949816	A/G	Comb, E1	4.27	11.10
	chrUnn_rand_2975131	C/A	Comb	4.21	10.96
				Total	36.50
R_1Dia					
	chrA07_22509933	G/A	E2	5.10	10.09
	chrA04_rand_54410	T/C	E2	4.42	8.80
	chrUnn_rand_4504809	A/G	E2	4.26	8.49
				Total	28.19
R_2Dia					
	chrAnn_rand_21292473	G/T	E2	5.13	10.14
	chrC09_35455232	G/T	E2	4.62	9.18
	chrA01_3788681	C/A	E2	4.58	9.10
				Total	28.42

4.4.4.4. Root dry weight (RDW)

PC26 were selected as the best fitted model for marker-RDW association in E1 and combined analysis (Table 4.2). However, MLM model Kinship+PC26, accounting for both relatedness and population structure was the best fitted model. Three markers,

chrC01_11247236, chrA07_12603394 and chrA07_rand_1125045 were detected at 0.01 percentile cut off in both E1 and combined dataset. Marker chrC01_11247236 gave highest peak with –log10 (*P*-value) of 6.69 in E1 and second highest peak with –log10 (*P*-value) of 5.26 in combined dataset. With one additional markers in each analysis, four markers in E1 accounted for 33.1% phenotypic variation, and four markers in combined datasets accounted for 32.6% of total phenotypic variation. In E2, four different markers from A01, A02 and A03 chromosome were found to be significantly associated with RDW at 0.01 percentile and altogether accounts for 8.3% of the phenotypic variation. Four candidate genes, *Auxin Signaling F-Box 4* (*AFB4, FBX14*) for marker chrC01_11247236, *Homeobox 53 (HB53)* for marker chrA07_rand_1125045, *GAST1* protein homolog 4 (*GASA4*) for marker chrA02_1172233 and *Tryptophan Aminotransferase Related 2 (TAR2)* for marker chrA01_7136448 were identified within the close proximity of the respective markers.

At less stringent 0.1 percentile cut off, four markers, chrA06_978037, chrA01_9006214, chrA07_22509933 and chrC08_17124940 were detected across all the datasets, environment E1, E2 and when data were combined (Table 4.4).

Table 4.4. Significant marker loci detected in both environment and datasets at 0.1 percentile tail P value cut off.

Trait & Markers	Env.	E1		E2		Comb	
		−log10 (*P*-value)	**R-Sq (%)**	**−log10 (*P*-value)**	**R-Sq (%)**	**−log10 (*P*-value)**	**R-Sq (%)**
RDW							
chrA06_978037	E1, E2, Comb	3.43	10.12	4.03	11.93	4.01	10.58
chrA07_9006214	E1, E2, Comb	3.95	11.57	3.44	1.80	4.09	11.57
chrA07_22509933	E1, E2, Comb	3.45	10.10	2.87	1.10	3.48	9.27
chrC08_17124940	E1, E2, Comb	3.47	10.22	3.54	2.40	3.81	10.10
RVS							
chrA06_2937010	E1, E2, Comb	3.61	8.57	3.15	7.63	3.95	9.18
chrA07_4128452	E1, E2, Comb	3.16	7.56	3.54	8.53	3.57	8.34

4.4.4.5. Root vigor score (RVS)

PC28 (in E1 and Combined) and Kinship + PC28 (in E2) were found to be the best fitted model for marker RVS association (Table 4.2). Seven markers passed the 0.01 percentile cut off across the two environments and in combined analysis. One marker with unknown position in the genome, chrCnn_rand_27194557, was detected in all three datasets and accounts for 2.3% - 14.4% of the phenotypic variation in E1, E2 and in combined dataset (Table 4.3). Two other markers from chromosome A01 and A04 were found significant in E1 and in combined datasets. The percentile of the phenotypic variation they explain is ranged from 11.1% - 18.6% across two datasets. Two candidate genes, *Indole Acetic Acid-Induced Protein 8 (IAA8)* 45.6 kbp downstream of the marker chrA04_11064427 (E1 & Comb) and *Phosphofructokinase 7 (PFK7*) 44.2 kbp downstream of marker chrA01_7949816 (E1 & Comb), were detected.

At 0.1 percentile cut off twelve markers were found to be significant in more than one datasets (Table 4.4). Among them, two markers, chrA06_2937010 and chrA07_4128452, were detected in both E1 and E2 environments and combined dataset.

4.4.4.6. Root diameters

We have collected root diameter only from 2016 or E2. Naïve model (accounted neither population structure nor relatedness) was found to be the best fitted model for both Soil level root diameter (R_1Dia) and bottom root diameter (R_2Dia). Three markers were significant at 0.01 percentile *P*-value cut off of 5.55E-05 for R_1Dia. Two of them were located on chromosome A04 (chrA04_rand_54410) and A07 (chrA07_22509933) and the other marker's position were unknown. The markers together explained 28.1% of the total phenotypic variation. Gene model BnaA04g27350D and BnaA04g27360D were found within and very close proximity, respectively, of the marker chrA04_rand_54410 (Table 4.5). Both of these gene model are very similar to *Arabidopsis* gene model AT3G60350 encoding *ARABIDILLO-2* protein. Another gene model was identified 35.7 kbp upstream of the same marker encoding *Indole-3-Acetic Acid Inducible 30 (IAA30)* in *Arabidopsis*.

In the case of R_2Dia, three significant markers were detected at *P*-value cut off 2.63E-05 at 0.01 percentile. These are chrAnn_rand_21292473, chrC09_35455232 and chrA01_3788681, and altogether explained 28.4% of the total phenotypic variation. Gene model BnaA01g07940D similar to *Arabidopsis* gene model AT4G28980 encoding *Cyclin-Dependent Kinase F; 1 (CDKF; 1*) was identified at 30 kbp downstream of the marker chrA01_3788681 (Table 4.5)

4.4.5. Marker validation

We have selected 18 most significant markers based on haplotype block and marker positions on chromosome for four traits, PRB, RVS, RL, RA and RDW, to validate the marker-trait association. Based on the predicted and observed phenotype, we were able to predict root traits with 12 markers for different root traits (Appendix Table A2a, A2b and A2c). Five markers for PRB, chrC04_2352222, chrC04_2352228, chrC04_2352267, chrA01_8813067 and

chrC03_1822934, successfully predicted the nature of PRB in 85% of the genotypes. Two markers, chrA04_5977698 and chrA01_7949816, predicted the root vigor in 75% of the genotypes. One RL marker, chrC03_4976549, was able to predict the nature of root length in 90% of the genotypes. Four markers for RA, chrC06_33610701, chrC06_33610722, chrC03_5436381 and chrC03_5436391, were able to predict the nature of root angle with 90% of accuracy.

4.5. Discussion

4.5.1. Root phenotyping

Recent advancement in high throughput phenomics has allowed plant scientists to phenotype root architectural traits efficiently with more accuracy. Several root phenotyping platforms were successfully used to phenotype different root architectural traits in different crops. Few examples are rhizotron in maize and sorghum (Singh et al., 2010; Lobet and Draye, 2013), hydroponics in corn and rice (Holloway et al., 2011; Clark et al., 2013b), transparent media in rice and soybean (Fang et al., 2009; Topp et al., 2013), shovelomics in maize (Trachsel et al., 2011), pouch and wick system in canola (Thomas et al., 2016). Most of the currently established phenotyping platforms are highly efficient for phenotyping root system at seedling stages when the root system is comparatively less complex in majority of the crops. In canola, we observed that root system in spring and winter growth habits does not show any significant variation at seedling stage, rather variation starts to arise from 40 days after planting and reaches maximum at 60 days after planting (Arifuzzaman and Rahman, 2017). Therefore, the variation of the root system difference between adult spring and winter canola plants may not be indicative by their root system at early growth or seedling stages. Root traits in seedling stage in other crops

may not be an accurate predictor of the mature root system or plant performance (Zhu et al., 2011).

In our earlier studies, we successfully phenotyped root vigor with a visual scoring system as an indicative trait for whole root system in 45-50 days old spring and winter canola (Rahman and McClean, 2013; Arifuzzaman et al., 2016). However, individual root architectural traits of an adult canola plant proved very difficult to phenotype with many of the existing phenotyping platform and imaging technologies. In this current study, we modified the "Mesocosms for Root Evaluation" procedure developed by Roots lab, University of Pennsylvania for maize root evaluation. Instead of using large PVC pipe in the original procedure, we used 40 cm x 10 cm pots which enable us to set up a large experiment with 224 genotypes with three replication in the greenhouse. We used sand and greenhouse soil mix at a ratio of 8:2. Higher amount of sand helped the root washing procedure while small amount of greenhouse soil mix improved the moisture holding capacity of the growing media. A plastic poly bag (perforated at bottom) was lined inside the pot before potting which allowed to bring out the intact root system with growing media before extraction. This modified method is very cost effective but efficient method for complex root system extraction from adult canola plant and easy phenotyping of different root architectural traits and can be used in other crops too.

4.5.2. Phenotypic distribution of canola root traits and relationship among them

In the current study we phenotyped seven root architectural traits in 2015 (E1) and 2016 (E2). The RL in both E1 and E2 showed a normal distribution, which indicates a polygenic control of the trait (Banga and Banga, 2009). Large mean differences were observed for RL and PRB between E1 and E2 datasets, inferring higher environmental interaction influencing the trait. The low heritability (0.3-0.31) for RL in both E1 and E2 supporting this statement.

Moderate heritability (0.44-0.62) was detected for RA and PRB in both environment and combined datasets. Higher root heritability for RA was also observed in durum wheat (Sanguineti et al., 2007). Root angle was suggested as a proxy trait for root length in rice (Kato et al., 2006) and sorghum (Singh et al., 2012) due to good heritability and easiness to measure comparing to root length. High heritability was observed for RDW and PRB in both environment (0.81-0.91). High heritability across the environment ensures the repeatability of the trait performance which is a prime criterion among the breeders during selection (Thomas et al., 2016). We observed high heritability for R_2Dia and moderate heritability for R_1Dia in E2.

Both RL and PRB were significantly positively correlated with RDW and RVS in the both environments and combined dataset indicating the role of RL and RDW in defining the whole root dry mass and root vigor. Indeed, higher root length with higher number of root branches will ultimately result vigorous and higher root mass. Interestingly, RVS is highly correlated with RDW in both environment and combined data set. This suggests that RVS can be a proxy trait for RDW (Rahman and McClean, 2013). Significant positive correlation were detected in R_1Dia vs R_2Dia indicates positive relationship between root diameters at two points. Both R_1Dia and R_2Dia is significantly positively correlated with RL, PRB, RDW and RVS which suggests that root diameter has an important impact on the overall root system architecture in canola. Significant positive correlation between different root traits except RA in each environment indicates their role in shaping the whole root system architecture and possibility of co-localization of the marker loci between root traits (Burridge et al., 2017).

4.5.3. Genome-wide association study

In the current study, we performed GWAS to identify useful marker loci associated with different root architectural traits. To detect the best marker-trait association, we tested six models

for each traits in each environment and combined dataset. The best fitted model for a trait in a specific environment was determined based on the lowest MSD value among the tested models (Mamidi et al., 2011). Therefore, we found different models to be best fitted for different traits in different environment which resulted different *P*-values in the best models for different traits in different environments (Table 4.2). Moghaddam et al. (Moghaddam et al., 2016) suggests that a single *P*-value cutoff for all traits in all environment might not be suitable to identify important marker loci. We bootstrapped the *P*-value 1,000 times and the markers at top 0.01 percentile of the empirical distribution considered as the significant markers (Mamidi et al., 2014; Moghaddam et al., 2016; Soltani et al., 2017). Possibility of identification of false positive marker association is high if the population was not corrected for structure and relatedness. However, the possibility of excluding true marker-trait association or false negative increases if the *P*-value cutoff is too stringent. Therefore, we also have taken into account the markers that pass a less stringent *P*-value cutoff at 0.1 percentile tail of the empirical distribution and appeared in all datasets E1, E2 and Comb.

We identified a total of 52 significant marker loci associated with different root traits at 0.01 percentile *P*- value cutoff in different environment and combined datasets. Five of the identified markers were not assigned on a known position of any chromosome based on the *B. napus* reference genome (Chalhoub et al., 2014). The remaining 48 marker loci were distributed among 17 *B. napus* chromosomes except A08 and C07. Chromosome A01, A02, A04, C03 and C06 contain a majority of majority of the marker loci associated with different root traits. Limited genetic research was conducted on the root system architecture of rapeseed/canola so far. Few reports were found in the previous literatures on the genetic loci controlling rapeseed/canola root system architectural traits in response to nutrient deficiency or abiotic

stresses. Shi et al., (2013) and Zhang et al., (2016) reported majority of the QTL on chromosome A03 for different root traits in response to low and high phosphorus (P) level. Zhang et al., (2016) also reported abundance of co-localized QTL for primary root number located on chromosome A04, C04, A08 and A09 in response to high and low phosphorus. Wan et al., (2017) reported three marker loci associated with root length under saline condition on chromosome A01, A03 and A06.

4.5.3.1. Root length (RL) and root angle (RA)

Tap root length (RL) is a crucial component in the root system architecture as it is directly related to depth of the soil zone that a plant can explore. This component become important especially when plant sense the drought condition and need to go deeper in the soil for moisture (Kong et al., 2014). Root system with higher root length is favorable for the plant to acquire nutrients like N, which tends to leach easily from the top soil (Wasson et al., 2012; Paez-Garcia et al., 2015). A QTL named *DEEPER ROOTING 1 (DRO1)* is described by Uga et al. (Uga et al., 2013) in rice which is controlled by auxin and enable plants go deeper for water under drought condition and yield higher. In the current study, total 10 marker loci associated with RL were detected in E1, E2 and combined dataset. Marker chrUnn_rand_2975131 appeared in both E1 and combined dataset as the highest peak. Majority of the markers are positioned mainly on the chromosomes of C genome, C02, C03, C04, and C06. Zhang et al. (Zhang et al., 2015) reported one marker loci on chromosome A03 associated with root length in canola under water stressed condition. Yang et al., (2010) reported two unique QTL on chromosome A03 and C02 for root length in response to low P. Wan et al., (2017) reported three marker loci associated with root length under saline condition on chromosome A01, A03 and A06.

Root angle in a root system architecture is mainly measured from the angle between the tap root and the primary roots. Root angle is a critical trait in a sense that its utility is different in response of different soil condition. Under low water regime in the soil, higher root angle will not be beneficial for the plant as it will create competition with the neighboring plants for water. Moreover, with higher angle root system spend its energy to explore relatively higher root zone when it would need to go deeper. Lynch, (2013) described the importance of "steep-cheap-deep" root system to increase water and nutrient uptake capacity of the root system in crop species. In the current study, eleven marker loci associated with root angle were identified on chromosome A01, A03, A06, A09, C02, C03, and C06.

We detected two marker loci (chrC03_4976549 and chrC03_5436381) within only 0.46 Mbp that are associated with RL and RA, respectively. This indicates a possibility of interrelated mechanism defining RL and RA in canola. Although we did not detected any correlation between RL and RA in canola, Kato et al., (2006) describes the association of root angle and root length in upland rice. Three candidate genes, *SAUR70* (early auxin response gene), *ABCG28* and *PIP2; 4* related to auxin transport were detected within the close proximity of these two markers.

4.5.3.2. Multiple markers on the same chromosomal regions are associated with PRB, RDW and RVS

Several markers within a close proximity on a chromosome were found to be associated with multiple root traits. Three markers on chromosome A01 (chrA01_7136448, chrA01_7949816 and chrA01_8813067, located on a close proximity of the physical map within 1.68 Mbp were associated with RDW, RVS and PRB, respectively (Fig 4.5a). The latter two markers were detected in both E1 and combined data set. Primary roots and lateral roots play the most important role in mining soil water and nutrients during vegetative and reproductive stage

in Arabidopsis and *B. rapa* (18). We have presented four candidate genes within this region (*TET7, ATMYB41, TAR2* and *PFK7*) related to various functions in root development, patterning and root epidermal cell differentiation. Therefore, we suggest, this region of chromosome A01 may be a hotspot controlling root architecture of canola. Arifuzzaman et al., (2016) reported one QTL for root vigor (RVS) on chromosome A01. Shi et al., (2011) also assigned one root related QTL under boron (B) deficiency on chromosome A01.

PRB, coming out from the tap root, are the integral part of the root system architecture giving an overall shape, density and angle of the whole root system in canola. Although we did not measure the lengths and diameters of the primary root branches, we noticed that these two factors are very important for lateral root growth and defining root system architecture in canola. We measured root dry weight and rate the overall root vigor in the current study which showed significant correlation to the primary root branch number. Therefore, we suggest that these traits can be a good indicator for primary root branch lengths and diameters or vise versa.

Three marker loci, chrA02_1108743, chrA02_1172218 and chrA02_1172233 resided within 64 kbp region on chromosome A02 and associated with both PRB and RDW (Fig 4.5b). We reported two candidate genes (*PIN8* and *GASA4*) related to auxin transport and GA mediated signaling pathway crucial in various root development procedure. Shi et al., (2011) identified several QTL for RDW under low and optimum boron (B) application. One of those QTL was assigned on A02. Additionally, two marker loci (chrA04_4928074 and chrA04_5977698) associated with RDW and RVS, respectively, are within 1.05 Mbp on chromosome A04. No candidate genes were detected within this region.

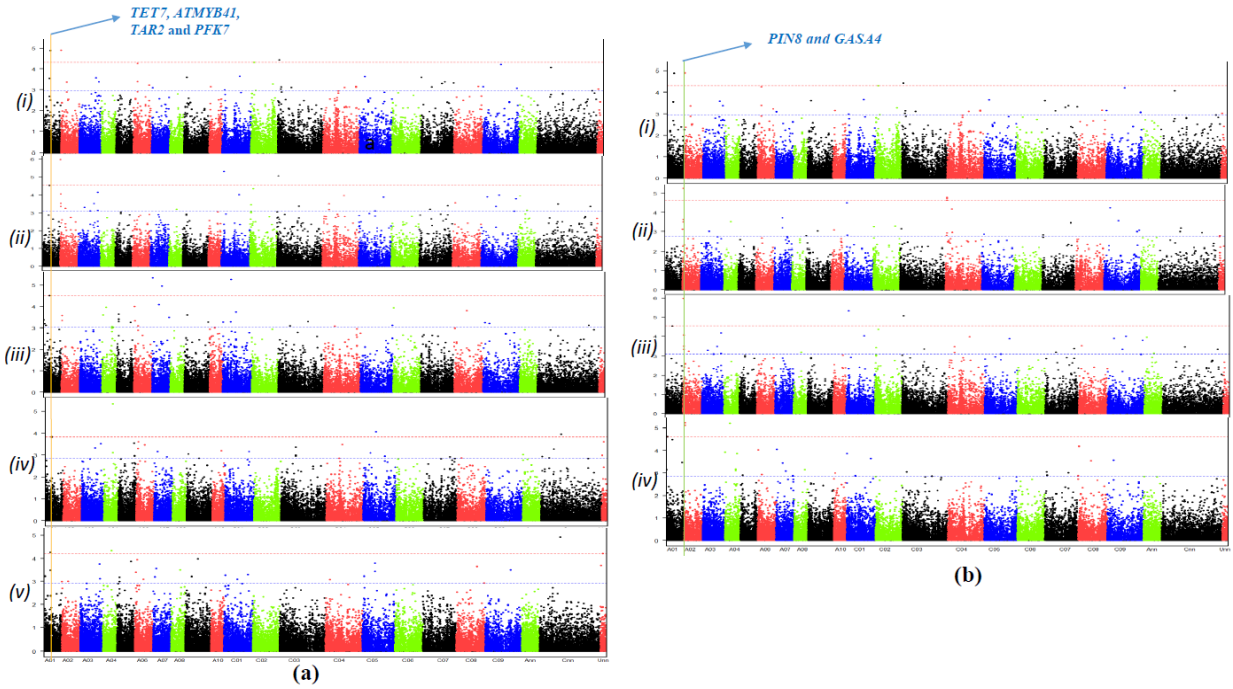

Figure 4.5. Manhattan plots showing co-localized markers associated with (a) PRB, RDW and RVS on chromosome A01 and (b) PRB and RDW on chromosome A02 where, a(i) = PRB in E1, a(ii) PRB in comb, a(iii) RDW in comb, a(iv) RVS in E1, a(v) RVS in comb, b(i) PRB in E1, b(ii) PRB in E2, b(iii) PRB in comb and b(iv) RDW in E2

4.5.3.3. Root diameters (R_1Dia and R_2Dia)

We took root diameter measurement at two points of the tap root, at the base of the tap root at soil level (R_1Dia) and 10 cm below the soil level (R_2Dia). Considering the significant positive correlation between root diameters and other root traits in the current study, we hypothesized that, root diameters at the tow points of the tap root are good indicators of the nature of the tap root growth on the top soil as well as other root parameters. Burridge et al., (2017) suggested that a tap root with higher diameter may be linked to the greater total xylem area in the taproot. Higher taproot diameter may help the plant to explore deeper soil domain to get higher water supply in a drought condition. Total six marker loci associated with R_1Dia and R_2Dia (3 loci each) distributed on chromosome A01, A04, A07 and C09 were identified.

4.5.4. Candidate genes

4.5.4.1. Candidate genes were identified on the same physical location of the markers

We have reported total 22 candidate genes involved in root growth and development within the 50 kbp upstream and downstream of the significant markers. Some of these candidate genes were located on the exact same physical location of the respective marker and warranted our attention. *P-glycoprotein 6* (*PGP6, ABCB6*) was detected on the RL marker chrC03_12098594. *PGP*s are the member of the ABC transporter superfamily and well regarded as the cellular and long distance auxin transporter (Geisler and Murphy, 2006) and involved in suppression of lateral root and root hair formation (Santelia et al., 2005). Marker loci chrA01_8813067 for PRB shares the same physical location with a gene encoding *Tetraspanin 7* (*TET7*) protein. Plant *TET* family proteins are believed to interact with auxin related processes and involved in leaf and root patterning (Wang et al., 2015). Two *Brassica* gene models (BnaA04g27350 and BnaA04g27360) were detected on the physical location of the marker locus chr04_rand_54410 associated with R_1Dia. Both of these gene model matched with *Arabidopsis* gene model AT3G60350 encoding *ARABIDILLO-2* protein. Overexpression of *ARABIDILLO-2* protein in *Arabidopsis* root resulted higher number of lateral roots and loss of function of this gene was found to decrease lateral root number (Coates et al., 2006). Therefore, this loci might be very important regarding lateral root formation.

Beside these, few other root related candidate genes were located within a very close distance of the respective markers. We have reported two candidate genes, *ABC-2* type transporter family (*ABCG28*) and *Plasma Membrane Intrinsic 2; 4* (*PIP2; 4*) on the chromosome C03 within the close proximity of the marker chrC03_5436381. *PIP2; 4* and another very similar protein *PIP4;4* were reported to be highly expressed in root hair and *pip4; 4* mutant

showed longer root hairs than the wild type plant in *Arabidopsis* (Lin et al., 2011). *ARGOSE*-like gene (*Auxin-Regulated Gene Involved in Organ Size Like*) was detected only 1 kbp downstream of the marker C04 which is expressed in root elongation zone and involved in organ growth (Markakis et al., 2012). Candidate gene *Homeo Box 53* (*HB53*) within very close proximity (4 kbp upstream) of RDW marker locus chrA07_rand_1125045 is a highly auxin inducible gene and regulate auxin/cytokinin signaling during root development (Son et al., 2004).

Table 4.5 Candidate genes for different root architectural traits within 50kb region at either side of the significant markers.

Trait	Locus	Env	BNA	AT	Distance*	Symbol (TAIR)	GO biological process (TAIR)
RL	chrC03_4976549	E2	BnaC03g10280D	AT5G20810	-23,984	SAUR70	Response to auxin
	chrC03_12098594	E2	BnaC03g22140D	AT2G39480	0	PGP6, ABCB6	Root hair elongation; Lateral root development; Auxin efflux transmembrane transporter activity; Acropetal auxin transport; Basipetal auxin transport
RA	chrC03_5436381	E1	BnaC03g11140D	AT5G60740	1,232	ABCG28	Transmembrane transport
			BnaC03g11160D	AT5G60660	14,338	PIP2;4	Root hair elongation; Response to abscisic acid
PRB	chrA01_8813067	E1,Comb	BnaA01g16850D	AT4G28050	0	TET7	Aging; Root and leaf radial pattern formation; Root morphogenesis
			BnaA01g16900D	AT4G28110	20,970	ATMYB41	Response to abscisic acid
	chrC03_1822934	E1,Comb	BnaC03g03700D	AT5G08590	-17,836	SNRK2	Response to salt stress
			BnaC03g03740D	AT5G08790	-2,144	NAC81, ATAF2	Response to JA and SA
	chrA02_1108743	E2	BnaA02g02480D	AT5G15100	-18,970	PIN8	Auxin efflux, Auxin homeostasis; Auxin polar transport; Auxin-activated signaling pathway
			BnaA02g02560D	AT5G15230	31,719	GASA4	Response to gibberellin stimulus; Gibberellic acid mediated signaling pathway
	chrC04_2352267	E2	BnaC04g03310D	AT2G44080	-1,779	ARGOSE-like, ARL	Organ growth; Multicellular organism development; Positive regulation of organ growth
RDW	chrC01_11247236	E1,Comb	BnaC01g16320D	AT4G24390	-37,392	AFB4, FBX14	Auxin-activated signaling pathway
	chrA07_rand_1125045	E1,Comb	BnaA07g37510D	AT5G66700	4,436	HB53	Response to auxin stimulus; Root development
	chrA02_1172233	E2	BnaA02g02560D	AT5G15230	-31,771	GASA4	Response to gibberellin stimulus; Gibberellic acid mediated signaling pathway
	chrA01_7136448	Comb	BnaA01g14030D	AT4G24670	-10,062	TAR2	Maintenance of root meristem identity; Primary root development; Response to ethylene; Lateral root development; Auxin biosynthesis Process, Positive gravitropism
RVS	chrA04_11064427	E1,Comb	BnaA04g13090D	AT2G22670	-45,699	IAA8	Auxin-activated signaling pathway; Lateral root formation; Negative regulation of lateral root development; Response to auxin,
	chrA01_7949816	E1,Comb	BnaA01g15420D	AT4G26270	-44,200	PFK7	Root epidermal cell differentiation

Table 4.5 Candidate genes for different root architectural traits within 50kb region at either side of the significant markers (Continued)

Trait	Locus	Env	BNA	AT	Distance*	Symbol (TAIR)	GO biological process (TAIR)
R_1Dia	chrA07_22509933	E2	BnaA07g32430D	AT1G76190	-30,013	SAUR56	Response to auxin
	chrA04_rand_54410	E2	BnaA04g27350D	AT3G60350	0	ARABIDILLO-2	Lateral Root Development
			BnaA04g27360D	AT3G60350	357	ARABIDILLO-2	Lateral Root Development
			BnaA04g27430D	AT3G62100	35,716	IAA30	Auxin-activated signaling pathway; Gravitropism; Response to auxin; Root development
R_2Dia	chrA01_3788681	E2	BnaA01g07940D	AT4G28980	-41,066	CDKF;1	Maintenance of root meristem identity

*negative values= genes are downstream of the marker, positive values= genes are downstream of the marker

4.5.4.2. Candidate genes are mostly related to auxin mediated root development

Auxin is the prominent among all plant growth hormones involving root growth and development (Petrasek and Friml, 2009). Auxin biosynthesis, transport and signaling are the major stages of auxin mediated root development. A number of genes, gene families and transcription factors are actively involved in these stages either to promote or repress root growth by controlling one or more root architectural traits. In the current study, auxin related candidate genes are dominant among the reported 22 candidate genes. These auxin related candidate genes are involved in either of the auxin biosynthesis, transporting or signaling process based on the TAIR website and existing literatures. The auxin related candidate genes identified in this study are shown in a diagram which explains the whole process of auxin biosynthesis, transport and signaling for root development (Fig. 4.6).

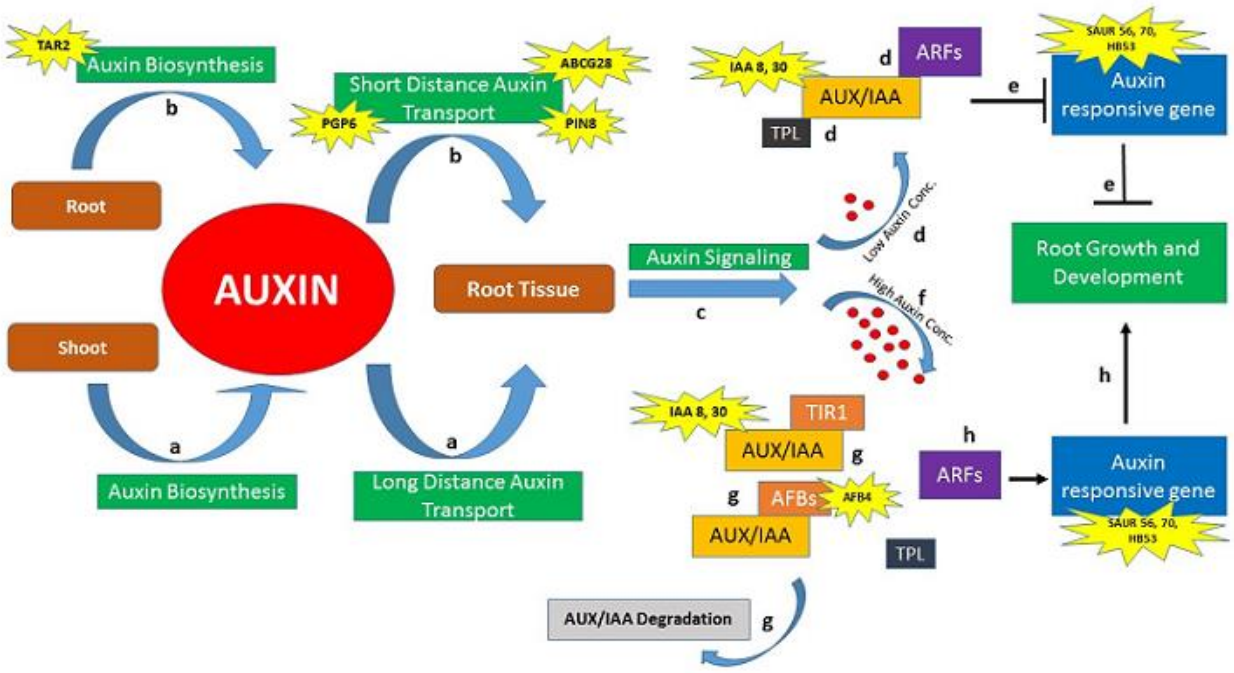

Figure 4.6. Diagram showing the auxin related candidate genes identified in the current study in auxin biosynthesis, transport and signaling process. (a) Auxin produced in shoots is transported to the root through phloem with long distance auxin transport system. (b) Auxin produced in roots and transported from cell to cell through efflux and influx auxin careers (PIN, PIP6 etc.). (c) Several auxin responsive genes involve in auxin signaling process. (d) With the low concentration of auxin, AUX/IAA, a transcriptional repressor, binds with AUXIN RESPONSIVE FACTOR (ARFs) with the help of a corepressor TOPLESS (TPL). Some of the ARFs act as transcriptional activator of different auxin responsive genes. (e) Due to binding with repressor (AUX/IAA), ARFs were unable to activate different auxin responsive genes controlling root growth and development. (f) Alternately when there is high concentration of auxin, (g) AUX/IAA binds with Transport INHIBITOR RESPONSE 2 (TIR2) or Auxin F-BOX PROTEINs (AFBs) and further degraded. (h) ARFs become free to transcriptionally activate auxin responsive genes promote root growth and development.

4.5.5. Marker validation

We were able to predict the nature of different root architectural traits with twelve markers in population consists of 20 germplasms with 75-90% accuracy. In most cases, the germplasms with major allele for a marker of a particular trait showed extreme high phenotypes. The markers were also be able to identify the germplasms with extreme low root traits. The root traits are mostly quantitative in nature and controlled with many genes of minor effect. Therefor

multiple markers need to be identified and validated for a single trait for successful prediction in a larger population.

4.6. Conclusion

In earlier studies, we phenotyped the root system on adult canola plants by a visual scoring system and dissect the root vigor of canola (Rahman and McClean, 2013; Arifuzzaman et al., 2016). In the current study, we modified a maize root system phenotyping platform to phenotype 55 days old canola root system for different root architectural traits. All the root traits were found positively correlated with each other except the root angle (RA). In marker trait association, we identified 52 significant marker loci associated with different root traits. Gene models within 50 kbp region upstream and downstream were blasted against *Arabidopsis* gene models. Twenty-two candidate genes related to the root traits were identified of which three of them were located exactly on three respective markers. Twelve markers were validated for four traits indicated that the markers could be used in marker-assisted selection breeding program.

CHAPTER 5. SHOVELOMICS FOR PHENOTYPING ROOT ARCHITECTURAL TRAITS OF RAPESEED/CANOLA (*BRASSICA NAPUS* L.) AND GENOME WIDE ASSOCIATION MAPPING[3]

5.1. Abstract

The root system architecture of spring and winter rapeseed/canola (*B. napus* L.) are different in terms of vigor and growth in later growth stages. In this study, 216 diverse genotypes were phenotyped for five different root architectural traits following shovelomics approach in the field condition during 2015 and 2016. A single nucleotide polymorphism (SNP) marker panel consists of 30,262 SNPs was used to conduct genome-wide association study (GWAS) to detect marker trait association. A total of 31 significant marker loci were identified at 0.01 percentile tail *P*-value cutoff for different root traits. Six marker loci for soil-level taproot diameter (R_1Dia), six loci for belowground taproot diameter (R_2Dia), seven loci for number of primary root branches (PRB), eight loci for root angle (RA), and eight loci for root score (RS) were detected in this study. Several markers associated with root diameters R_1Dia and R_2Dia were also associated with PRB and RS. Significant phenotypic correlation between these traits was observed in both environments. Therefore, taproot diameter appears to be a major determinant of the canola root system architecture and can be used as proxy for other root traits. Fifteen candidate genes related to root traits and root development were detected within 100 kbp

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upstream and downstream of different significant markers. This is the first report on the genetics of *B. napus* root architectural traits in the field condition.

Key words: GWAS, shovelomics, root traits, *Brassica napus*

5.2. Introduction

Brassica napus L. is an amphidiploid species (AACC) developed from natural hybridization of two other diploid *Brassica* species, *B. rapa* (AA) and *B. oleracea* (CC) (U, 1935) . Oil from older genotypes of rapeseed (*B. rapa* and *B. napus*) was high in erucic acid and glucosinolate, and the use was limited to lamp-fuel and lubricants. Modern day canola was developed in 1970s by lowering the erucic acid (< 2%) and glucosinolate content (<30µm/gm) by a team of University of Manitoba plant breeders through conventional breeding approach. Rapeseed/canola oil is now regarded as one of the healthiest edible oils worldwide (Lin et al., 2013). It is the second largest oilseed crop in terms of global production after soybean (Foreign Agriculture Services, USDA, 2017).

Various genotypes of canola have spring, winter and semi-winter growth habit that is based on growing season and a vernalization requirement for flowering (Ferreira et al., 1995; Rahman and McClean, 2013). Beside their flowering time difference, spring and winter types are distinct in terms of other morphological characteristics. Winter type canola plants have larger leaves, wider stem diameters, higher plant height, and more vigorous root system with higher yield compared to spring canola. According to Kebede et al., (2010), the spring and winter canola are genetically diverse from one another. With superior morphological and yield traits, winter canola can be a great resource for improving spring canola yield and morphology (Arifuzzaman et al., 2016).

The root system has several major functions such as supplying water and nutrients, acting as food and water storage unit, and anchoring the plant to the soil (de Dorlodot et al., 2007). The spatial arrangement of the whole root system in the soil is referred as its root system architecture (Lynch, 2007). Different components of a root system i.e. root length, primary root branch, lateral root branch, root density, root diameter, root angle, total root surface etc. (Kuijken et al., 2015) define the root system architecture. Different root architectural traits respond to the soil microenvironment differentially and can be modified according to the plant needs. Therefore, the root system plays a significant role in adaptation of plants under abiotic stress conditions. According to Lynch, (2007), This has lead plant scientists (Lynch, 2007) to suggest a greater focus on the root system modification to enhance crop adaptation under different environmental stresses to increase levels of crop production. Manipulation of root system architectural traits of crops to grow in nutrient deficient or drought condition can significantly enhance the yield (Wasson et al., 2012; Kuijken et al., 2015)

The root system has remained the "the hidden half" throughout the last century in the field of plant breeding and crop improvement. Plasticity of the root system and difficulties in phenotyping root traits has discouraged breeder from focusing on root traits for crop improvement. Recent advancement in genetic mapping and molecular marker technologies encouraged breeders to emphasize on the root architectural traits for crop improvement through marker assisted selection (Clark et al., 2011). Nevertheless, large scale, highly accurate and efficient phenotyping of root traits are essential for an ideal mapping study (Canè et al., 2014). Recent technological advances in the field of phenomics allows plant breeders to successfully evaluate the root architecture of different crops from younger seedlings in controlled environment. There are very few methods for large scale high throughput phenotyping for the

root architectural traits in the field. Shovelomics is one of the popular methods of root system phenotyping in the field. Shovelomics involves digging up the root system with shovel from the field, washing them and measure the root traits with the help of a phenotyping board and/or imaging technology (Trachsel et al., 2011).

Genome wide associate studies (GWAS) were first implemented to study human diseases as an alternate to family-based mapping strategies (Begum et al., 2015). Eventually, it become very popular among plant scientists to identify quantitative trait loci. A GWAS is performed with a set of diverse germplasms which enable us to capture all the historical recombination events within a germplasm panel (Zhao et al., 2014; Rahaman et al., 2017) and is highly efficient in identifying common alleles controlling a complex trait. Single nucleotide polymorphism (SNP) markers are now the most popular marker due to their low cost and ease of development. Researchers are now able to develop genetic maps with higher marker density with the help of abundantly generated SNPs (Luo et al., 2016).

Genome wide association mapping is performed to successfully identify QTL and molecular markers associated with different root traits in different crops. However, most of these studies were performed under controlled condition. There are only few GWAS examples of phenotyping for root architectural traits in the field; i.e. rice with PVC pipe method (Li et al., 2017), rice with basket method (Lou et al., 2015), and cowpea with shovelomics (Burridge et al., 2017). In the current study, we adopted the shovelomics approach described by (Trachsel et al., 2011) to phenotype genetically diverse *B. napus* germplasms for different root architectural traits, and to identify genomic regions associated with different root architectural traits of rapeseed/canola (*Brassica napus* L.)

5.3. Materials and methods

5.3.1. Plant Materials and experimental design

A total of 216 *B. napus* genotypes of diversified origin (Appendix Table 5.1) were planted in North Dakota State University Agricultural Experiment Station at Carrington, N. D. in 2015 and 2016. The soil type of the area is Heimdahl silt loam with pH of ~6.5 and OM of ~2.7 All the three growth habit types of canola/rapeseed were included in the germplasm panel: spring (n=85), winter (n=92) and semi-winter (n=39). The germplasm accessions were obtained from Germplasm Resource Information Network (GRIN) of USDA-ARS. Germplasms were planted as randomized complete block design with two replications in each year.

5.3.2. Phenotyping

Root architectural traits were phenotyped with the Shovelomics approach described by (Trachsel et al., 2011). Phenotyping was done at 50 days after planting. The total phenotyping procedure is divided into three major steps (Fig 5.1). Three representative plants per plot were selected based on plant height and general appearance and excavated them with a shovel. The root system in 50 days old canola plant is very complex and grows deep in the soil horizon. Therefore, we excavated the root system only to capture the top 10 cm of the root system and phenotyped the root traits. The root sample is then washed. All three excavated plants with their root systems were zip tied together, tagged and brought to the washing area. The roots were washed first with soapy water followed by a wash with normal water. After 5-10 minutes of air-drying, data measurements were taken. Data was collected on number of primary root branches (PRB), lateral root angle (RA), taproot diameter at soil level (R_1Dia), belowground taproot diameter at 10 cm below the soil level (R_2Dia) and a visual scoring of the root system (RS) (Table 5.1) RA and the first 10 cm length of the taproot were measured with a phenotyping board

labeled with angle and length measurement. Diameters were taken with slide calipers at the soil surface position and 10 cm down from the first data point. RS were recorded with a visual scoring system based on general appearance of the root system, and scored from 1-5, where score “1” represents the weakest root system with the thinnest taproot and fewest branch roots, score “2” represents weak root system with thin taproot and less root branches, score “3” represents root system with intermediate taproot diameter and intermediate root branches, score “4” represents root system with higher root vigor, thick taproot and root branches and score “5” represents very vigorous root system with the thickest taproot and higher number of root branches (Fig 5.2).

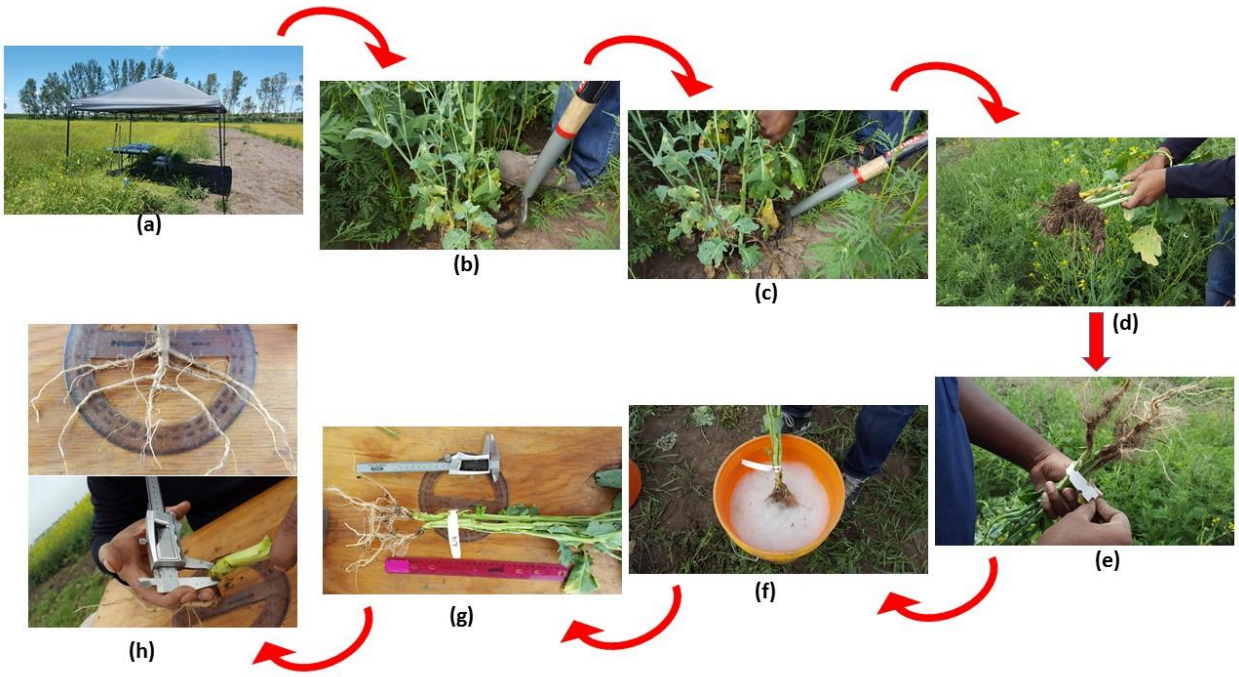


Figure 5.1. Shovelomics in phenotyping root system architectural traits of canola. (a) Work station in the field; (b) Selecting representative plants and excavating; (c) Excavating; (d) Clearing dart; (e) Tagging; (f) Washing; (g) Phenotyping board; (h) Data collection

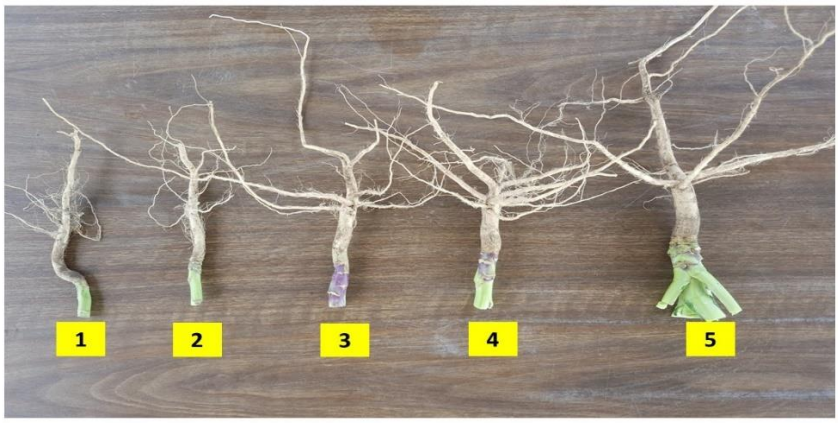


Figure 5.2. Visual root scoring with 1-5 scale, where (1) is the weakest root system with low taproot diameter and primary root branches and (5) is the strongest root system with high taproot diameter

5.3.3. Statistical Analysis

Data from three representative plants per plot were averaged and were analyzed 2015 (E1) and 2016 (E2) separately in SAS 9.3 (SAS Institute Inc., USA). Means were used to construct the analysis of variance (ANOVA) for all traits except RS, for which the medians were calculated and used. If the ratio of the effective error variance for each trait is less than 10 folds, data from both environments were combined and used for the GWAS analysis (Tabachnick and Fidel, 2001; Elias and Manthey, 2016). Pearson correlation coefficients were calculated in SAS 9.3 for all traits except RS for which spearman correlation coefficient were calculated. Shapiro-Wilk test was conducted in SAS 9.3 for all traits in both E1 and E2 and combined dataset. Entry mean basis heritability was calculated in SAS 9.3 for all traits in both E1, E2, and combined data set. RS data was available for only one replication in E1, and therefore the heritability was not calculated for RS in E1.

Table 5.1. Root Architectural traits, their abbreviation and description used in this study

Trait	Abbreviation used	Description
Soil level taproot diameter	R_1Dia	Taproot diameter at the soil level
Belowground taproot diameter	R_2Dia	Taproot diameter at 10 cm below the soil level
Primary root branch number	PRB	Number of primary root branches came out from the taproot at top 10 cm of the root system
Root Angle	RA	Angle between the two top most primary root branches came out from the Taproot
Root Scoring	RS	Visual scoring of the root excavated root system

5.3.4. Genotyping

A total of 366 genotypes from diverse origin and sources were planted in the greenhouse in 2013 (Michalack et al, Unpublished). Young leaf tissues were collected from each germplasms and freeze dried. DNA were extracted by using Qiagene DNeasy kit (Qiagen, CA, US) following the manufacturers protocol. After extraction, DNA were quantified, checked for quality, optimized and sent to Institute of Genomic Diversity (IGD), Cornell University for Genotyping by Sequencing (GBS). GBS libraries were prepared by using *ApeKI* single cutter enzyme following the protocol described by Elshire et al., (2011). Libraries were sequenced with Illumina GAII sequencer and 100 bp single end sequenced GBS data were aligned by using BWA-MEM (Li, 2013). VarScan (Koboldt et al., 2012a) were used for SNP calling. SNPs were imputed for missing allele by using FastPHASE (Scheet and Stephens, 2006) and finally 42,575 SNPs were obtained. For the current experiment, we used the SNP data with minor allele frequency greater than 0.05 on 216 genotypes. The name of each SNP marker consists of chromosome number and physical position of that marker. For example, a SNP marker located on 123456 bp of chromosome C04 was named as “C04_123456”.

5.3.5. Marker-trait association

Association mapping was performed using GAPIT R-based program (Lipka et al., 2012). Number of principle components (PC) that collectively explaining 25% of the variation in the population was determined by principle component analysis (PCA) in R 3.4.1 using *prcomp ()* function (Price et al., 2006). An pairwise kinship coefficient matrix was calculated to account for individual relatedness by using the EMMA algorithm (Kang et al., 2008) embedded in GAPIT. A total of four models were tested to detect the marker-trait association, (1) a null general linear model or naïve model accounting for neither population structure nor kinship; (2) a general linear model accounting for population structure as a fixed effect using the number of PC accounting for 25% of the variation (PC-25%); (3) an efficient mixed model association or EMMA accounting for kinship in the population; and (4) a mixed linear model accounting for both population structure and kinship, PC-25% + EMMA, All the models were compared on a rank based mean square deviation (MSD) value, and the model with the lowest MSD value was selected as the best model (Mamidi et al., 2011). Significant markers were declared based on the *P*-value of the markers at lower 0.01 and 0.1 percentile tail of an empirical distribution obtained by 10,000 bootstraps (Mamidi et al., 2014). Manhattan plots were created by using *mhtplot ()* function in R statistical software package gap (Zhao, 2007). Log likelihood ratio based R^2 or R^2_{LR} (Sun et al., 2010) was calculated in genAble package in R.4.1 (Aulchenko et al., 2007) for the most significant markers in the best models to determine the phenotypic variation explained by those markers.

5.3.6. Candidate gene

B. napus gene models within 100 kbp upstream and downstream of the significant markers were taken into account for candidate gene search. Protein sequences from the gene

models were blasted against TAIR 10 protein database to determine the gene annotation. Genes associated with root development were identified based on the Gene Ontology terms (GO terms) from TAIR website and gene functions found in previous literatures.

5.4. Results

5.4.1. Phenotypic Distribution

Phenotypic variation was observed among the germplasm panel in both environments and when the data was combined (Figure 5.3). The range between maximum and minimum observations in 2015 for R_1Dia (22.88), R_2Dia (8.15), PRB (13.80) and RA (96.79) was the higher than those of 2016 (Table 2). The CVs of R_1Dia (27%), R_2Dia (44%), PRB (31%) and RS (31%) in 2015 were higher than the CVs for the respective traits in 2016. Relatively higher CV was observed for R_2Dia across both environments and the combined dataset (31-44%). The lowest CVs (6-9%) were observed for RA across both years and with the combined datasets ranging. High family-based mean heritability was detected for R_1Dia in both years and for combined dataset (0.60-0.79) (Table 5.2). High heritability was detected for R_1Dia in 2015 (0.83), but moderate heritability was detected in 2016 (0.60) and the combined dataset (0.67). A similar trend of high heritability in 2015 (0.72) and moderate heritability in 2016 (0.56) and the combined dataset (0.39) were found for PRB. Very low heritability was observed for RA in combined dataset (0.33). RS heritability in 2015 and combined dataset found moderate ranging from (0.46-0.58).

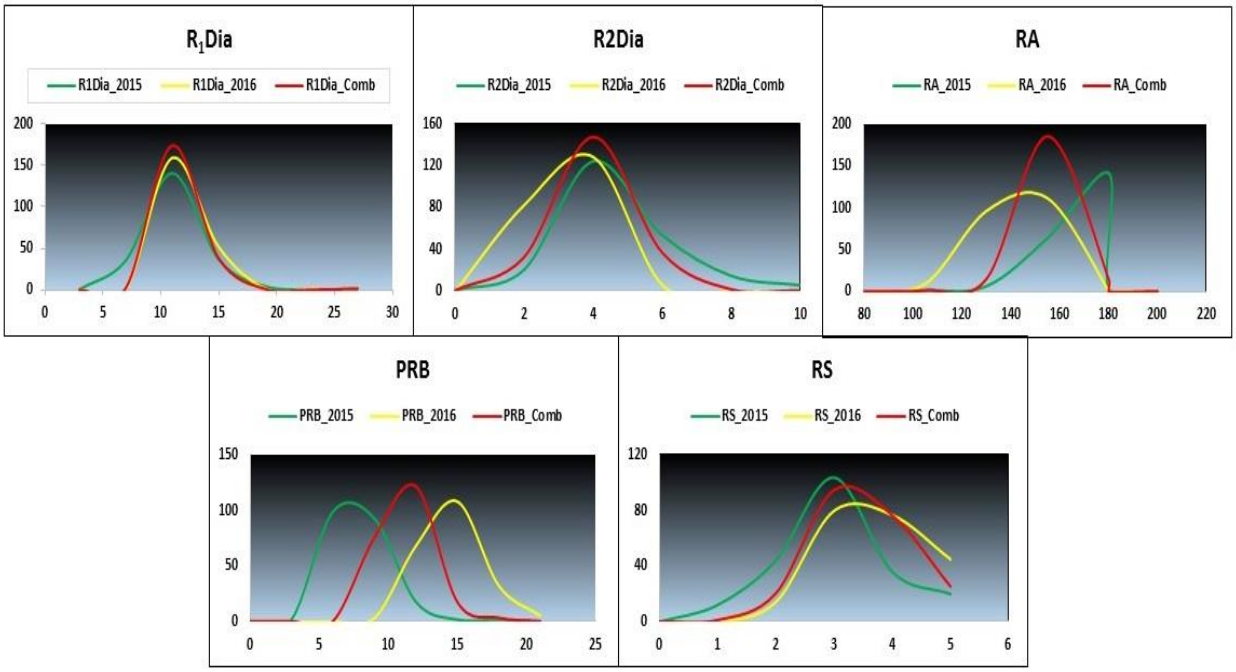


Figure 5.3. Phenotypic Distribution of different root architectural traits in 2015, 2016 and combine dataset.

Table 5.2. Phenotypic variation in different root architectural traits

Traits	Env.	Unit	Mean (±std)	Range	CV (%)	Shapiro-Wilk test p value	h^2 (family mean basis)
R_1Dia							
	2015	mm	9.35 (±2.57)	22.88	27%	0.0001	0.79
	2016	mm	10.08 (±1.84)	13.34	18%	0.0001	0.60
	Comb	mm	9.71 (±1.92)	17.27	19%	0.0001	0.67
R_2Dia							
	2015	mm	3.61 (±1.61)	8.15	44%	0.0001	0.83
	2016	mm	2.28 (±0.71)	4.45	31%	0.0001	0.32
	Comb	mm	2.95 (±0.98)	4.86	33%	0.0001	0.41
PRB							
	2015	number	6.49 (±2.07)	13.80	31%	0.0001	0.72
	2016	number	13.09 (±2.15)	11.70	16%	0.0378	0.56
	Comb	number	9.79 (±1.64)	11.2	17%	0.0001	0.39
RA							
	2015	degree	157.18 (±13.31)	96.79	8%	0.0001	0.43
	2016	degree	128.70 (±11.36)	63.75	9%	0.0001	0.60
	Comb	degree	142.94 (±9.08)	56.53	6%	0.0001	0.33
RS							
	2015	Scoring	2.95 (±0.93)	4.00	31%	0.0001	---
	2016	Scoring	3.47 (±0.87)	3.75	25%	0.0001	0.58
	Comb	Scoring	3.33 (±0.78)	4.00	23%	0.0001	0.46

5.4.2. Correlation among the traits

Significant positive correlation ($r = 0.57$-0.65, $P < 0.001$) was detected between R_1Dia and R_2Dia in all datasets (Fig 5.4). Both the root diameters, R_1Dia and R_2Dia, were significantly ($P < 0.001$) positively correlated with RS in all datasets. PRB and R_1Dia were also significantly ($P < 0.001$) correlated with each other in all environment. Very low or negative nonsignificant correlation were detected between RA and all other traits.

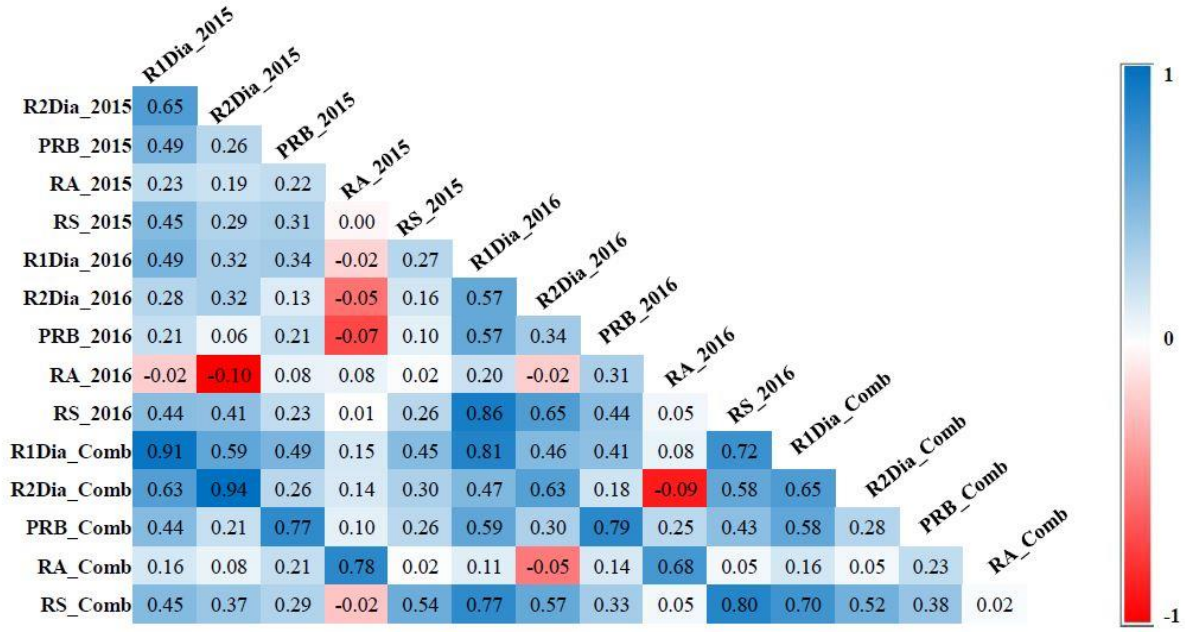


Figure 5.4. Correlation among the root architectural traits in 2015, 2016 and combined dataset

5.4.3. Genotypic data and principle component analysis

Originally 366 genotypes were genotyped from which 42,575 single nucleotide polymorphism (SNP) markers were obtained as described by Michalack et al. (Unpublished). In this current study, a subset of 216 genotypes were used. From the original panel, 30,262 markers were retained for the panel evaluated here after correcting for MAF > 5% and were used finally in this current study. Principle component analysis was performed to control for population structure. The first two principle components grouped the population into three continuous subpopulations (Fig 5.5). The first three principle components accounted for 25% of the population variation and were included in the association analysis as general linear model.

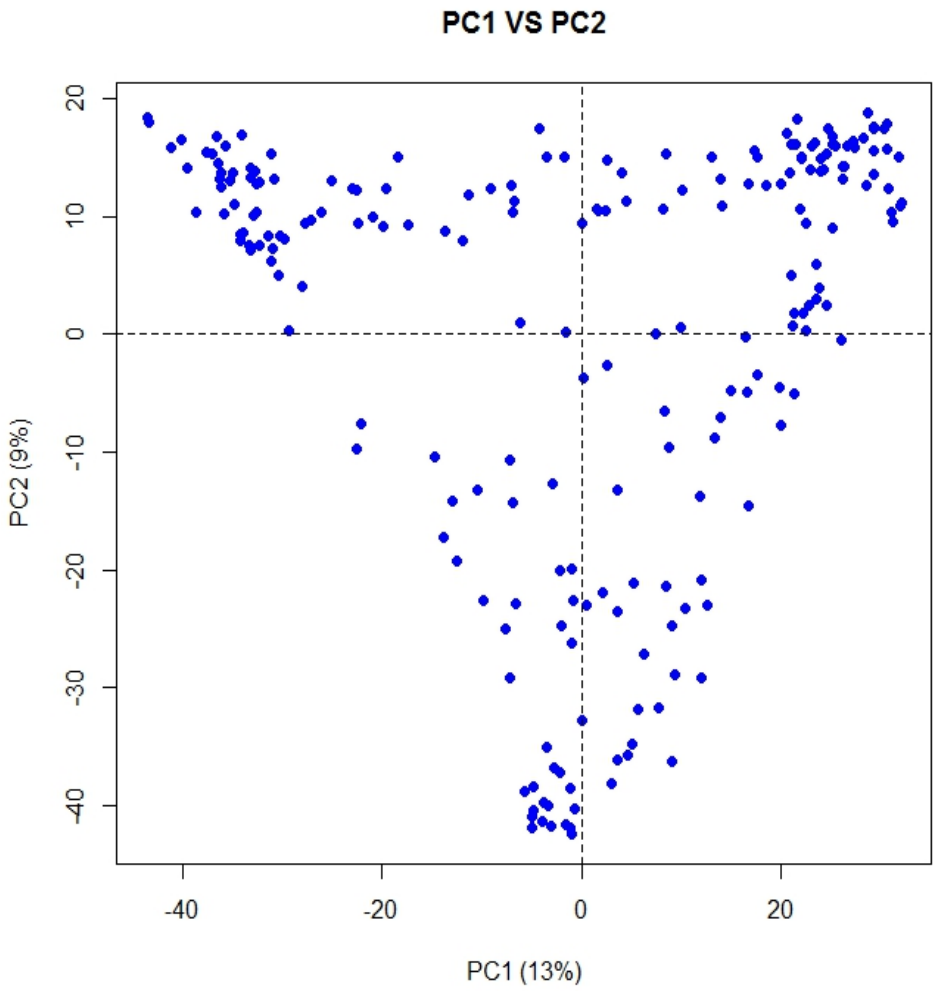


Figure 5.5. Principle Component graphs showing the distribution of the population used in this study.

5.4.4. Marker-trait association

Four models were tested for each trait in each year and with the combined over the two years to detect best SNP marker/trait association. Among the models, the best model was selected based on the lowest MSD value (Table 5.3) (Mamidi et al., 2014; Soltani et al., 2017). The *P*-values of the best model for a trait in a particular dataset (2015, 2016 or combined) were bootstrapped 1,000 times, and the *P*-value at 0.01 percentile of the empirical distribution were set as the cut-off for that particular trait in the respective dataset. Highly stringent *P*-value cut off

increases the chance of eliminating false positive or the false association. However, it also increases the chance of excluding false negative or true association. To reduce the chance of excluding true association or falls negative, we fixed another less stringent *P*-value cut off at 0.1 percentile of the empirical distribution (Moghaddam et al., 2016). Only the markers that were found to be appear repeatedly for a trait in both years and the combined dataset were considered to be a significant association and were reported here. Candidate genes were searched 100 kbp at either side of a significant marker at 0.01 percentile cut off only.

Table 5.3. Best models based on lowest MSD value with cut off P-value at 0.01 and 0.1 percentile tail for each trait in each environment and in combined data set.

Traits	Year	Best Model	MSD value	*P*-value cut off at 0.01 percentile tail	−log10 (P-value) at 0.01	*P*-value cut off at 0.1 percentile tail	−log10 (P-value) at 0.1
R_1Dia							
	2015	EMMA	1.18E-05	2.64E-04	3.58	1.86E-03	2.73
	2016	EMMA	6.48E-05	4.96E-05	4.30	2.33E-03	2.63
	Comb	EMMA	2.17E-05	1.25E-04	3.90	1.82E-03	2.74
R_2Dia							
	2015	PC3	2.95E-04	5.60E-06	5.25	2.08E-03	2.68
	2016	PC3+EMMA	4.14E-04	3.62E-04	3.44	2.59E-03	2.59
	Comb	PC3	3.03E-04	2.20E-04	3.66	2.09E-03	2.68
PRB							
	2015	EMMA	2.34E-05	3.37E-04	3.47	1.63E-03	2.79
	2016	PC3	5.60E-04	1.19E-04	3.92	9.17E-04	3.04
	Comb	PC3	2.63E-04	2.56E-04	3.59	1.31E-03	2.88
RA							
	2015	Naïve	1.19E-05	2.84E-04	3.55	1.41E-03	2.85
	2016	EMMA	1.02E-03	1.49E-03	2.83	4.72E-03	2.33
	Comb	PC3+EMMA	5.29E-06	1.60E-04	3.80	8.52E-04	3.07
RVS							
	2015	EMMA	2.01E-06	3.95E-04	3.40	1.41E-03	2.85
	2016	Naïve	1.62E-05	5.50E-04	3.26	2.11E-03	2.68
	Comb	EMMA	7.69E-04	9.90E-04	3.00	4.77E-03	2.32

5.4.4.1. Soil level taproot diameter (R_1Dia)

The mixed linear model accounting for relatedness in the population was the best fitted model in both years and combined over the two years (Table 5.3). Three markers, chrA01_rand_2039614, chrC01_rand_171065, and chrC04_4933647 were found to be associated with R_1Dia in 2015 at the 0.01 percentile *P*-value cut off level (Table 5.4). The markers together explained 39.2% of the phenotypic variation. Marker locus chrA01_rand_2039614 from 2015 was also detected in 2016 and the combined dataset. Marker locus chrC01_rand_171065 from 2015 was also detected in combined dataset. In 2016, two other markers chrC02_30907736 and chrA09_32034416 explaining 16.2%, and 16.8% of the phenotypic variation, respectively were detected. Two root related candidate genes *STRUBBLIG- receptor family 3 (SUB*) was detected nearby the marker locus chrA09_32034416 (2016) (Table 5.5). Marker chrC05_rand_678307 was detected in combined dataset and two candidate genes, *Cyclin-dependent kinase 2;3* (*CYC2; 3*) and *P-glycoprotein 11* (*PGP 11*) were detected nearby physical location of the marker.

5.4.4.2. Bottom taproot diameter (R_2Dia)

A general linear model using the first three principal component was selected as the best model for R_2Dia in 2015 and combined data (Table 5.3). Three markers loci, chrA08_18428869, chrA08_18428839 and chrA08_18441370, within a very close physical distances on chromosome A08, were identified as the significant markers at 0.01 percentile *P*-value cut off in both 2015 and the combined dataset (Table 5.4). The markers altogether explained 13.2% and 9.8% of the phenotypic variation in 2015 and combined dataset, respectively. The fact that the three loci only marginally account for more of the phenotypic variation suggest they are linked to the same causative locus. Two candidate genes, *AUXIN RESISTANT 3* (*AXR3*) and *Cryptochrome 2* (*CRY2*), were identified in the same region of chromosome A08 (Table 5.5). A

mixed linear model, PC3+EMMA, accounting for both population structure and relatedness was selected as the best model in 2016. Three unique marker loci, chrC05_7043010, chrA10_5618818 and chrA09_6247199, were found to be significantly associated with R_2Dia in 2016. They altogether accounted for 33.8% of the phenotypic variation (Table 5.5).

5.4.4.3. Primary root branches (PRB)

EMMA, accounting for kinship in the population, was found to be the best model for PRB in 2015 (Table 5.3). On the other hand, PC3, accounting for population structure, was identified as the best fitted model in 2016 and combined dataset. In 2015, marker loci chrC01_rand_171065, chrC04_24065408 and chrC03_3328679, were identified as the significant markers associated with PRB at 0.01 percentile *P*-value cut off and accounts for 37.4% of the phenotypic variation (Table 5.4). *GAST1*, a homolog of *GASA4* was detected at 53 kbp upstream of the marker locus chrC03_3328679 (Table 5.5). One of the 2015 marker loci, chrC01_rand_171065, was also detected in combined dataset and explained 9.2% phenotypic variation. Two other unique markers, chrA06_2653673 and chrA08_4600588, were also found to be significantly associated with PRB in combined dataset. Marker locus chrA06_2653673 was also found to be associated with PRB in both E1 and E2 with a less stringent at 0.1 percentile *P*-value cut off. A lateral organ boundaries (LOB) domain family (LBD1) gene was detected at 30 kbp upstream of the marker locus chrA06_2653673. At 0.01 percentile *P*-value cut off, two markers chrC05_7907786 and chrA08_6097421, were identified in 2016 and explained 16.9% of the phenotypic variation. *Aminotransferase-like, plant mobile domain family protein (MAIN)* was detected at 87 kbp downstream of the marker locus chrC05_7907786.

Table 5.4. Significant markers for different root architectural traits in 2015, 2016 and combined datasets at 0.01 percentile

Trait	Markers	Year. (0.01)	Year. (0.1)	Allele	MAF	−log10 (*P*-value)		R-Sq (%)
R_1Dia								
	chrA01_rand_2039614	2015, 2016, Comb		C/T	0.14	4.76		18.49
	chrC01_rand_171065	2015, Comb		G/C	0.05	4.64		17.86
	chrC04_4933647	2015, Comb		A/T	0.11	3.58		13.36
							Total	39.24
	chrA01_rand_2039614	2016, 2015, Comb		C/T	0.14	4.37		11.65
	chrC02_30907736	2016	Comb	A/G	0.13	4.35		16.21
	chrA09_32034416	2016		A/T	0.16	4.30		16.79
							Total	31.99
	chrA01_rand_2039614	Comb, 2015, 2016		C/T	0.14	5.81		22.93
	chrC01_rand_171065	Comb, 2015		G/C	0.05	4.49		17.79
	chrC05_rand_678307	Comb	2015, 2016	C/T	0.26	3.99		14.45
							Total	45.43
R_2Dia								
	chrA08_18428869	2015, Comb		T/C	0.20	5.99		12.25
	chrA08_18428839	2015, Comb		A/G	0.20	5.75		11.54
	chrA08_18441370	2015, Comb		G/T	0.21	5.25		10.36
							Total	13.15
	chrC05_7043010	2016		T/A	0.15	3.80		14.82
	chrA10_5618818	2016		C/T	0.48	3.57		15.76
	chrA09_6247199	2016		G/A	0.07	3.44		13.56
							Total	33.78
	chrA08_18428869	Comb, 2015		T/C	0.20	4.63		9.27
	chrA08_18428839	Comb, 2015		A/G	0.20	4.49		8.78
	chrA08_18441370	Comb, 2015		G/T	0.21	4.12		7.91
							Total	9.76
PRB								
	chrC01_rand_171065	2015, Comb		G/C	0.05	5.25		18.04
	chrC04_24065408	2015	Comb	T/A	0.12	3.98		18.00
	chrC03_3328679	2015		A/T	0.18	3.79		17.31
							Total	37.37
	chrC05_7907786	2016		T/C	0.36	4.27		8.20
	chrA08_6097421	2016		A/T	0.45	3.93		10.45
							Total	16.90

Table 5.4. Significant markers for different root architectural traits in 2015, 2016 and combined datasets at 0.01 percentile (Continued).

Trait	Markers	Year. (0.01)	Year. (0.1)	Allele	MAF	−log10 (*P*-value)		R-Sq (%)
	chrA06_2653673	Comb	2015, 2016	C/T	0.13	4.59		9.15
	chrC01_rand_171065	Comb, 2015		G/C	0.05	4.04		8.30
	chrA08_4600588	Comb	2015	C/A	0.09	3.85		7.56
							Total	22.45
RA								
	chrA02_18466347	2015, Comb		A/G	0.49	4.17		7.31
	chrA09_8677526	2015		G/A	0.13	4.16		7.29
	chrA03_25132124	2015		G/C	0.13	3.79		6.49
							Total	21.09
	chrC03_36049749	2016		C/T	0.35	2.88		10.90
	chrA03_18531995	2016		A/C	0.10	2.86		10.30
	chrC09_8316001	2016		T/A	0.132	2.83		9.12
							Total	29.75
	chrA02_18466347	Comb, 2015		A/G	0.49	4.75		16.23
	chrC06_35857438	Comb		C/T	0.06	3.94		16.23
	chrA02_22847077	Comb		G/A	0.09	3.87		12.83
							Total	32.85
RS								
	chrC01_11345932	2015		C/T	0.22	4.89		17.34
	chrC09_19217839	2015		C/T	0.43	3.53		13.05
	chrC05_13423686	2015		G/A	0.24	3.41		12.08
							Total	28.48
	chrC09_23510860	2016		C/T	0.15	3.46		5.84
	chrA01_rand_2039614	2016, Comb		C/T	0.14	3.43		5.78
	chrC05_rand_678307	2016	Comb	C/T	0.26	3.36		5.63
							Total	17.25
	chrA09_32034416	Comb		A/T	0.16	3.84		14.53
	chrA09_32034411	Comb	2016	A/T	0.16	3.39		12.64
	chrA01_rand_2039614	Comb, 2016		C/T	0.14	3.07		10.66
							Total	20.46

5.4.4.4. Root angle (RA)

The naïve model, accounting for neither the population structure nor kinship, was the best model in 2015 for RA (Table 5.3). A mixed linear model including kinship and PC3+EMMA were the best model in 2016 and combined dataset, respectively. Marker locus, chrA02_18466347 was detected as the most significant marker associated with root angle in both 2015 and combined dataset (Table 5.4). This marker explained 7.3% and 16.2% of the total phenotypic variation in 2015 and combined dataset, respectively. The other two marker loci found to be significantly associated with RA in 2015 are chrA09_8677526 and chrA03_25132124 and accounted 7.3% and 6.5% of the total phenotypic variation, respectively. Candidate gene *SABRE*-like protein (*SABRE*) and auxin-responsive *GH3* family were detected at 58 kbp upstream and 34 kbp downstream, respectively, of the marker locus chrA09_8677526 (Table 5.5). Candidate gene *Cytokinin response factor 4 (CRF4)* and *Tetraspanin 7 (TET7)* were detected at 5 kbp and 36 kbp downstream, respectively, of the marker locus chrA03_25132124. Three marker loci, chrC03_36049749, chrA03_18531995 and chrC09_8316001, were detected for RA in 2016, and they altogether explained 29.8% of the total phenotypic variation. Two unique marker loci, chrC06_35857438 and chrA02_22847077, were detected in combined dataset. *Auxin responsive factor 17 (ARF17)* was detected at 99 kbp downstream of the marker locus chrC06_35857438 associated with RA in combined dataset. Another candidate *GA requiring 3 (GA3)* were detected at 36 kbp downstream of the marker chrA02_22847077 associated with RA in combined dataset.

5.4.4.5. Root score (RS)

A mixed linear model including kinship was found to be the best model in 2015 and the combined analysis (Table 5.3). Three marker loci, chrC01_11345932, chrC09_19217839 and

chrC05_13423686, were found to be significantly associated with RS in 2015 at 0.01 *P*-value cut off (Table 5.4). The markers together explained 28.5% of the total phenotypic variation. Candidate gene *Cytokinin responsive factor 6* (*CRF6*) was detected at 89 kbp upstream of the marker locus chrC09_19217839 (Table 5.5). In combined dataset, three additional marker loci, chrA09_32034416, chrA09_32034411 and chrA01_rand_2039614, explained 20.4% of the total phenotypic variation were detected. A candidate gene *STRUBBLIG -receptor family 3 (SUB)* was located on chromosome A09, close to the marker loci chrA09_32034416 and chrA09_32034411. Marker locus chrA01_rand_2039614 was also appeared in 2016 and explains 5.8% of the total phenotypic variation. Two other marker loci, chrC09_23510860 and chrC05_rand_678307, were also detected in 2016, and explained 5.8% and 5.6% of the total phenotypic variation, respectively. Candidate gene *P-glycoprotein 11 (PGP 11)* was detected 0nly 10 kbp upstream of the marker locus chrC05_rand_678307. Naïve model was selected as the best fitted model for 2016 based on the lowest MSD value.

Table 5.5. Candidate genes for different root architectural traits within 100kbp region at either side of the significant markers.

Trait	Locus	BNA gene model	AT match	Distance (Kbp)*	Name (TAIR)	*Symbol (TAIR)*	GO biological process (TAIR)
R_1Dia	chrA09_32034416	BnaA09g47730D	AT1G11130	-91.7	*STRUBBELIG-receptor family 3*	*SUB, SCM, SCRAMBLED*	Root meristem specification
	chrC05_rand_678307	BnaC05g49810D	AT1G15570	80.3	*Cyclin-dependent kinase 2;3*	*CYCA2;3, CYCLIN A2;3*	Lateral Root Formation
		BnaC05g01280D	AT1G02520	9.9	*ABC* transporter family, *P-glycoprotein 11*	*PGP 11*	Auxin efflux transmembrane transporter activity; Basipetal auxin transport
R_2Dia	chrA08_18428869	BnaA08g27770D	AT1G04250	18.5	*AUXIN RESISTANT 3*	*AXR3, IAA17*	Auxin-activated signaling pathway, Response to auxin
	chrA08_18441370	BnaA08g27870D	AT1G04400	1.9	*Cryptochrome 2*	*CRY2*	Root development
PRB	chrC03_3328679	BnaC03g06850D	AT5G15230	53.9	*GAST1 protein homolog 4*	*GASA4*	Response to gibberellin stimulus, Gibberellic acid mediated signaling pathway
	chrC05_7907786	BnaC05g13870D	AT1G17930	-87.7	*Aminotransferase-like, plant mobile domain family protein*	*MAIN, MAINTENANCE OF MERISTEMS*	Meristem development
	chrA06_2653673	BnaA06g04390D	AT1G07900	30.7	*Lateral organ boundaries (LOB) domain family*	*LBD1*	Organ Boundary Speciation, Lateral root formation
RA	chrA09_8677526	BnaA09g14910D	AT1G58250	58.4	*SABRE*-like protein, *HYPERSENSITIVE TO PI STARVATION 4*	*HSP4, SABRE*	Negative regulation of ethylene-activated signaling pathway, response to ethylene
		BnaA09g15010D	AT1G48670	-34.2	*auxin-responsive GH3 family*	*auxin-responsive GH3 family*	Response to Auxin
	chrA03_25132124	BnaA03g48910D	AT4G27950	-5.2	*Cytokinin response factor 4*	*CRF4*	Cytokinin-activated signaling pathway, Ethylene-activated signaling pathway,

Table 5.5. Candidate genes for different root architectural traits within 100kbp region at either side of the significant markers (Continued).

Trait	Locus	BNA gene model	AT match	Distance (Kbp)*	Name (TAIR)	*Symbol (TAIR)*	GO biological process (TAIR)
	chrC06_35857438	BnaC06g38360D	AT1G77850	-99.5	*Auxin responsive factor 17*	*ARF17*	Auxin-activated signaling pathway
	chrA02_22847077	BnaA02g31830D	AT5G25900	-67.4	*GA requiring 3*	*GA3*	Gibberellic acid mediated signaling pathway, Gibberellin biosynthetic process
RS	chrC09_19217839	BnaC09g21990D	AT3G61630	89.6	*Cytokinin response factor 6*	*CRF6*	Cytokinin-activated signaling pathway, Ethylene-activated signaling pathway,
	chrC05_rand_678307	BnaC05g49810D	AT1G15570	80.3	*Cyclin-dependent kinase 2;3*	*CYCA2;3, CYCLIN A2;3*	Lateral Root Formation
		BnaC05g01280D	AT1G02520	9.9	*ABC* transporter family, *P-glycoprotein 11*	*PGP 11*	Auxin efflux transmembrane transporter activity; Basipetal auxin transport
	chrA09_32034416	BnaA09g47730D	AT1G11130	-91.7	*STRUBBELIG-receptor family 3*	*SUB, SCM, SCRAMBLED*	Root meristem specification

*negative values= genes are downstream of the marker, positive values= genes are upstream of the marker

5.5. Discussion

5.5.1. Shovelomics in *B. napus*

Plant breeders have historically modified the above ground morphological plant architecture of a crop to improve productivity. One of the greatest examples is the green revolution that developed high yielding dwarf verities in wheat (Vergauwen and De Smet, 2017). Modifying shoot features like branch angle and leaf angle are also regarded as the potential to improve the productivity of corn (Gong et al., 2015) and sorghum (Truong et al., 2015). This allowed to increase planting density, the capture of more sunlight, and increased photosynthetic efficiency of the crops. Similarly, the below ground root architectural traits can also be used as a target for improvement and optimization according to the necessities based on soil environment. Several studies were conducted to phenotype root system architectural traits and to map associated loci in many crops such as rice (Courtois et al., 2013), wheat (Canè et al., 2014), maize (Pace et al., 2015), cowpea (Burridge et al., 2017), rapeseed (Wang et al., 2017) etc. A majority of these studies adopted root system phenotyping strategy that are feasible for controlled environment using soil or other growing media and phenotyped the root system of young seedlings. The root systems of young seedlings grown in hydroponics or soil in the controlled environment may not be indicative of the architecture that forms in the actual field condition (Zhu et al., 2011). Several researchers mentioned the importance of monitoring root architectural traits over the whole growth period in the soil condition as the root system architecture changes greatly over time (Hund, 2010; Kumar et al., 2012; Passioura, 2012; Colombi et al., 2015). Therefore, phenotyping the root system under all stages of growth in the field condition is immensely important, and high throughput phenotypic techniques should be developed to overcome the difficulties in exploring root system in the field.

Unfortunately, very few high throughput platforms for phenotyping the root system architecture in the field have been developed to date. Shovelomics is a high throughput root phenotyping system first described by Trachsel et al., (2011). However, only a few studies have adopted this shovelomics approach to characterize the root system in field conditions in different crops such as maize (Bucksch et al., 2014; Colombi et al., 2015; York and Lynch, 2015), common bean and cowpea (Burridge et al., 2016). Although Trachsel et al., (2011) proposed manual measurement of the data in the original shovelomics approach, most studies used image based technologies during data collection. Burridge et al., (2017) conducted a GWAS analysis for cowpea root architectural traits with the phenotypic data collected with shovelomics approach. In the current study, we also adopted the field-based shovelomics technique to phenotype 216 *B. napus* genotypes in multiple years and used that date for a GWAS analysis. This is the first report of shovelomics to characterize the root architectural traits in *B. napus*.

In the current study, we have phenotyped five root traits and conducted a GWAS analysis. Based on a stringent cut off at lower 0.01 percentile of bootstrap *P*-values, 31 marker loci were associated with different root traits in 2015, 2016 and in the combined datasets. Markers were distributed in all the *B. napus* chromosomes except A04, A05, A07, C07 and C08. Five marker loci were detected on chromosome A08 controlling R_2Dia and PRB. Four marker loci controlling various root traits were detected on chromosome A09. Multiple co-localized marker loci were detected on chromosome A08 and A09 controlling primary root number under high and low P level (Zhang et al., 2016).

5.5.2. Phenotypic distribution and correlation among the root architectural traits

In the current study, five different root architectural traits R_1Dia, R_2Dia PRB, RA and RS in 2015 and 2016 were phenotyped. Analysis was performed for both years and the combined

dataset. Population means were relatively constant across the years for R_1Dia, R_2Dia and RS. In contrast, population means for PRB and RA varied across years indicating a genotype x environment effect controlling these traits. Development of lateral root branches are highly responsive to the soil nitrogen (N) and phosphorus (P) condition and water regime. On the basis of range, phenotypic variation within the population was always higher in 2015 to 2016. This also indicates the environmental influences controlling the root traits. Entry mean basis heritability for different traits varied with environments. High heritability values were noted for R_1Dia, R_2Dia and PRB in 2015. However, the heritability values decreased to moderate levels for all these traits in 2016 suggesting high environmental variation between these two environments.

Significant and high correlation were observed among the traits within the same year and in the combined datasets. R_1Dia and R_2Dia are highly correlated in both environment and combined dataset. A high correlation was also observed between PRB and R_1Dia. This is expected as the taproot having high diameter will have more surface area to grow more primary and lateral root branches and the root will be more vigorous. This statement is supported further as a high positive correlation were observed between R_1Dia and RS and between PRB and RS. Visual root scoring was conducted based on the overall appearance of the root system on top soil considering diameter and root branches. Low but significant positive correlation were detected between R_2Dia and PRB indicating lesser role of bottom tap root diameter on root branches. Interestingly, we did not observe any significant correlation between RA and other root traits in the current study. A lack of correlation between other traits and RA suggests that RA might be an independent trait which depends on soil environment rather than other root characteristics in *B. napus.*

5.5.3. Root diameters can be a proxy for other root traits

In the current study, multiple marker loci associated with R_1Dia were also found to be associated with RS and PRB. Marker loci chrA01_rand_2039614 and chrC05_rand_678307 were found to be associated with both R_1Dia and RS and detected in multiple datasets (Fig 5.6). Candidate gene *CYC2; 3* and *PGP11* were identified close to the marker chrC05_rand_678307. *CYC2* is involved in lateral root formation and triple *CYC2* mutant gives rise to defective lateral roots compared to the wild type (Vanneste et al., 2011). The *PGP* family of proteins are members of *ABC* transporter superfamily and few directly act as auxin efflux career in basipetal auxin transport and affect lateral root and root hair formation (Santelia et al., 2005). Two other significant marker loci, chrA09_32034411 and chrA09_32034416, are physically close and were found to be associated with RS and R_1Dia, respectively (Fig 5.6). Candidate gene *STRUBBELIG-receptor family 3 (SUB)* were found in the same region on chromosome A09. *SUB* is expressed in the root throughout the root development process and controls root meristem specification and epidermal root hair specification in *Arabidopsis* (Savage et al., 2013; Kwak and Schiefelbein, 2014). Marker locus chrC01_rand_171065 was associated with PRB and R_1Dia. In addition to these, marker loci chrC05_7043010 and chrC05_7907786 are located within 9 kbp and are associated with both R_2Dia and PRB, respectively. Candidate gene *Aminotransferase-like, plant mobile domain family protein* or *MAIN* or *MAINTENANCE OF MERISTEM* were found close to this marker locus. Experimental evidences suggests mutants of *maintenance of meristem* have a defective stem cell niche in *Arabidopsis* roots (Wenig et al., 2013). *MAINTENANCE OF MERISTEM* homolog *MAIN-like 1* mutant have short primary root branches (Ühlken et al., 2014).

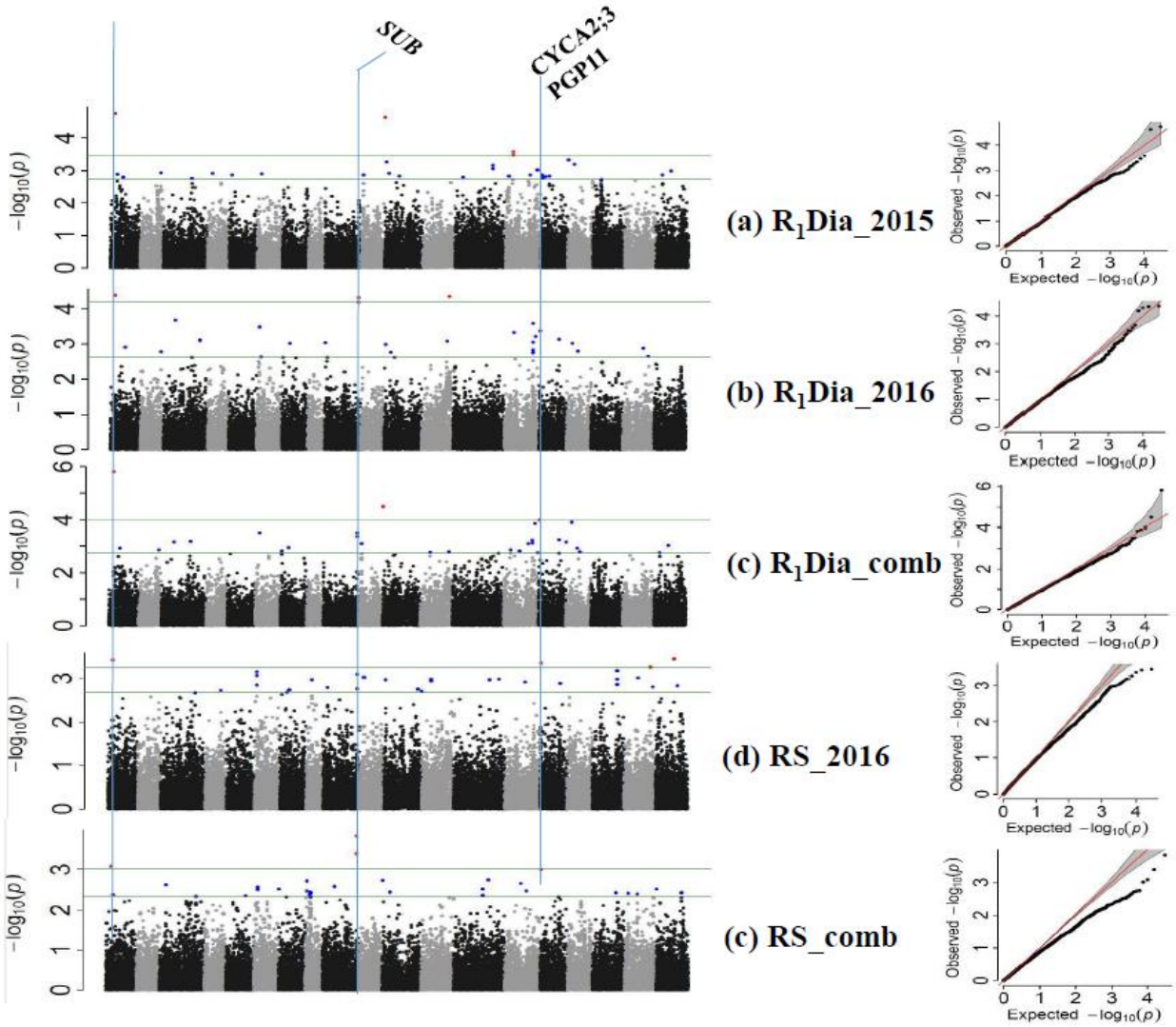


Figure 5.6. Manhattan plot and QQ plot showing the common significant markers associated with R_1Dia and RS. (a) R_1Dia _2015, (b) R_1Dia_2016, (c) R_1Dia _combine, (d) RS_2016 and (e) RS_combine

Common marker loci or a region associated with R_1Dia, RS, PRB and R_2Dia is expected because we took PRB data from the top 10 cm of the root system and RS scoring was based on visual appearance of the root system consisting of both PRB and taproot diameters. In our phenotypic study, R_1Dia, PRB and RS were highly correlated with each other in both years. Higher soil level tap root diameter has higher root surface area to accommodate higher root lateral branches and root hairs. A root system with high tap root diameter is correlated with

higher xylem area in cowpea (Burridge et al., 2017) which may transport water and nutrients from the root to the shoots more efficiently. A high phenotypic correlation between R_1Dia and R_2Dia also indicates that root diameters may have a positive role in defining root length. Burridge et al. (2017) suggested that taproot diameter at 10 cm may be related to the plants ability to explore a deeper soil horizon in drought conditions for cowpea. Considering all these aspects, we propose that tap root diameter in *B. napus* can be a good indicator for other root architectural traits and may serve as a proxy trait for the whole root system. Importantly, the soil level taproot diameter R_1Dia can be measured without destroying the plant.

5.5.4. Significant marker loci associated with same trait in multiple datasets

The marker locus chrA01_rand_2039614 was significantly associated with R_1Dia in both 2015, 2016 and combined datasets. Marker locus chrC01_rand_171065 was also significantly associated with R_1Dia in 2015 and the combined dataset. Marker locus chrC05_rand_678307 was identified in the combined dataset for R_1Dia at 0.01 *P*-value cut off. However, with a less stringent 0.1 percentile *P*-value cut off, these marker loci were found to be significant in both 2015 and 2016. For R_2Dia, three closely located marker loci on chromosome A08, chrA08_18428869, chrA08_18428839 and chrA08_18441370 were found to be significantly associated in both 2015 and combined datasets. Two candidate genes, *AXR3* and *CRY2*, were detected in the same region of chromosome A08 (Fig 5.7). *Auxin Resistant 3* or *AXR3/IAA17* overexpression caused defective roots in *Arabidopsis* (Kim et al., 2006). Knox (2003) reported that *AXR3/IAA17* inhibits root hair initiation and elongation. The relative abundance of *AXR3/IAA17* and *SHY2/IAA3* (gene which induce root hair formation and elongation) in a cell is the determinant of root hair formation in *Arabidopsis*. Overexpression of *CRY2* in blue light results in reduced primary root elongation (Canamero et al., 2006).

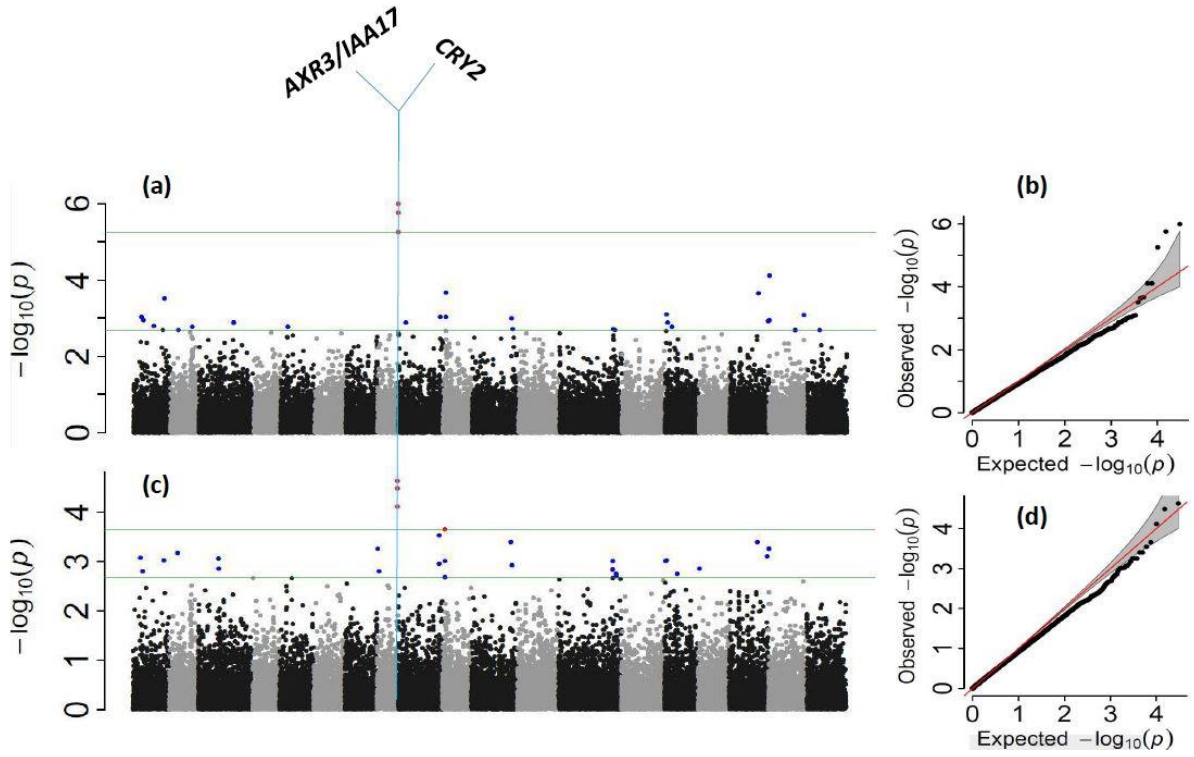


Figure 5.7. Manhattan plot and QQ plot showing the results of marker trait association of R_2Dia. (a) Manhattan plot of R_2Dia in 2015, (b) QQ plot of R_2Dia in 2015, (c) Manhattan Plot of R_2Dia in combine dataset, (d) QQ plot of R_2Dia in combine dataset

For PRB, one marker locus chrC01_rand_171065 was repeatedly significant in 2015 and the combined dataset. Marker locus chrA06_2653673 was significantly associated with PRB in combined dataset was also detected in 2015 and 2016 at the less stringent 0.1 percentile *P*-value cut off. *Lateral Organ Boundary domain 1* (*LBD1*) was detected close to this marker locus. Constitutive expression of *LBD1* in the *Medicago trancatula* root system subjected to salt stress regulates the overall root architecture (Ariel et al., 2010). Primary lateral roots appear from the taproot near the soil surface and are necessary for nutrient acquisition. Lateral roots in plants are highly responsive to the soil microenvironment. It has been reported that the length of lateral root was increased under low N condition in *Arabidopsis*, and lateral root density was increased in P deficient soil conditions in rice (Desnos, 2008; Gruber et al., 2013; Kong et al., 2014). Therefore,

the significant markers identified for PRB in this study may be useful in future marker assisted breeding program to select for improved nutrient acquisition.

Root angle is an important trait for root architecture where a narrower root angle allows the root to penetrate deeper into the soil horizon which reduces the competition between the neighboring plants. In addition, the plant will have an excess of moisture from the deeper soil horizon too. Seven marker loci associated with root angle (RA) were identified in 2015, 2016 and the combined dataset. Among them the marker locus chrA02_18466347 was found to be significant in both 2015 and the combined dataset. Root score was measured visually on the basis of overall appearances of the root system and eight marker locus were identified related to root score (RS). Marker locus chrA01_rand_2039614 was associated with RS in both 2016 and the combined datasets.

5.6. Conclusion

We followed the shovelomics approach to phenotype five root architectural traits and use the data for GWAS of these root traits with SNP markers. In this study, a total of 31 significant marker loci associated with different root traits were identified. Several marker loci were found to be associated with multiple root traits and appeared in multiple environments. It appears that tap root diameter in rapeseed/canola may be a determinant of the total root system in canola and can be a proxy trait for other root architectural traits. Tap root diameter can be measured without uprooting the plant from soil. Fifteen candidate genes related to different root traits were detected within 100 kbp downstream and upstream of different significant markers.

CHAPTER 6. TRANSCRIPTOME ANALYSIS SUGGESTS CYTOKININ AND GIBBERELLIN SIGNALING MAY ACCOUNT FOR DIFFERENCES BETWEEN SPRING AND WINTER RAPESEED/CANOLA (*BRASSICA NAPUS* L.) ROOT DEVELOPMENT[4]

6.1. Abstract

Spring and winter canola growth habits are different from each other in terms of yield and plant morphology. Spring type canola produces significantly smaller and less vigorous roots compared to winter type canola at the same growing stage. We conducted this experiment to identify the genetic variation, gene regulatory networks and cross talk among phytohormones that may responsible for the difference between the root system of the two growth types. We analyzed transcriptomes from the root samples collected from two spring and two winter types canola at two time points, 30 and 60 days. A total of 169,646 transcripts were analyzed. Among them, 582 and 555 transcripts were found to be significantly differentially expressed between spring and winter types at 30 and 60 days, respectively. Several auxin responsive family genes were significantly differentially expressed between spring and winter types at both time points but no distinct pattern of differential regulation among auxin response or signaling genes was observed in either of the growth types. This would be most consistent with the hypothesis that some other non-auxin mediated root growth regulation might be responsible for the root system

[4] [4] This chapter has been submitted to BMC Plant Biology journal for publication. This article is co-authored by Muhammad Arifuzzaman, David Horvath and Mukhlesur Rahman. MA and MR formulated the experiment. MA conducted the experiment, collected the data, analyzed the data and wrote the manuscript. DH heled in data analysis. DH and MR reviewed the manuscript.

differences between the two types. Type-A and type-B *ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) genes and gene sets involved in cytokinin signaling pathway were up-regulated in spring types compared to winter types at 60 days. This would be consistent with higher cytokinin activity in spring types root system at later growth stage which has proven to inhibit root growth. In addition, several gibberellin responsive and gibberellin signaling gene sets were also upregulated in spring types compared to winter types at 60 days. We observed an elongated root system with fewer root branches in spring type compared to winter type at 60 days in this study. Root elongation but inhibited lateral root formation is consistent with a role of gibberellin and what we observed in the spring types root system architecture. Therefore, we suggest that cytokinin and gibberellin may play a major role in reduced root growth in spring canola in the current study although gibberellin mediated tap root elongation may be occurred. Extensive gene expression data generated in this research will further assist to understand the natural variation of root system in canola growth habits.

Key Words: Transcriptomics, Auxin, Gibberellin, Cytokinin

6.2. Introduction

Canola (*Brassica napus* L.) stands for "***CAN***adian ***O***il ***L***ow ***A***cid" was developed in 1974 by lowering the harmful erucic acid and glucosinolate content from seed. Canola (known as rapeseed in Europe) gained substantial popularity worldwide for its healthy attributes. In North America, canola is predominantly grown in Canada and in some parts of United States. North Dakota alone holds over 80% of the US canola acreages in last few years (NASS, USDA, 2016).

There are three types of growth habits of canola; winter type, spring type and semi winter type canola. Winter and spring type canola are the two extreme opposite in terms of shoot and

root morphology and belong to two distinct genetically diverse groups (Rahman and Kebede, 2012). Winter type canola has vigorous root system with higher taproot diameter, taproot length, and a larger number of branches compared to those of spring canola (Arifuzzaman and Rahman, 2017)

Root system in plants plays versatile roles in plant development including water and nutrient absorption from the soil and providing anchorage to the plant (Arifuzzaman et al., 2016). A very complex gene network and cross-talk between different growth hormones play critical role from the point of radicle initiation to the formation of different types of root cells via root meristematic zone, elongation zone and differentiation zone (Overvoorde et al., 2010). In mature plant, auxin is produced in shoot and transported to the area of root tissue by phloem (only shoot to root transport) via various auxin transporter carriers where it regulates root growth and development (Goldsmith, 1977; Tsurumi and Wada, 1980; Müller et al., 1998; Ljung et al., 2001; Petrasek and Friml, 2009). In *Arabidopsis thaliana, like-auxin1 (LAX1)* and *Pinformed (PIN)* proteins are the two auxin influx and efflux carries, respectively, and both play an important role in cell to cell auxin transport (Bennett et al., 1996; Swarup et al., 2001; Geisler and Murphy, 2006). In the presence of auxin, *Aux/IAA* repressors are degraded by auxin receptors which facilitate the *Auxin Responsive Factors (ARF).* ARFs then activate the auxin responsive genes that likely lead to lateral root formation (Okushima et al., 2007; Fukaki and Tasaka, 2009; Petricka et al., 2012). Another phytohormone, cytokinin (CK), has cross-talk with auxin transport and signaling pathways and promotes cell differentiation (Saini et al., 2013). *Short-hypocotyl2 (SHY2)* gene, a major auxin repressor gene, is activated by cytokinin, which in turn, hampers auxin transport and signaling process via downregulation of transporter *PIN* genes resulting reduced root growth (Dello Ioio et al., 2008; Chapman and Estelle, 2009). Auxin, on

the other hand, has the ability to induce degradation of the SHY2 protein to restore activities of *PIN* genes to promote root growth (Benjamins and Scheres, 2008). Gibberellic acid plays an important role in the auxin-CK cross-talk by repressing the CK mediated inhibitory effects on root growth (Greenboim-Wainberg, 2005; Moubayidin et al., 2010). Abscisic acid (ABA), in contrast, inhibits the expression level of auxin receptors in the presence of nitrate, and thus impairs the degradation of *Aux/IAA* repressors leading to reduced lateral growth (Signora et al., 2001; Vidal et al., 2010).

Elucidation of complex mechanisms of root growth and development is important because root system architecture can be exploited to deploy tolerance against abiotic stresses and increase yield. Comprehensive knowledge on transcriptomic gene profiles in plant root system should assist in our understanding of the regulatory gene networks and complex physiological pathways that shape the root system architecture. Transcriptome analysis is proven to be a successful tool in studying the transcriptome and decoding differential gene expression profile in root system of various plant species i.e. *Arabidopsis* (Lan et al., 2012; Vidal et al., 2013; Begara-Morales et al., 2014; Li et al., 2015), *B. napus* (Yong et al., 2014; Dun et al., 2016), rice (Zhai et al., 2013; Yoo et al., 2017), maize (Stelpflug et al., 2016), soybean (Song et al., 2016) etc. A majority of these studies were conducted to discover the root growth mechanisms and role of different hormonal pathways controlling root system architecture under either abiotic stress or nutrient deficit condition and in the seedling stage.

In our previous research, we observed no significant difference between root parameters of spring and winter canola until 30 days after planting. Rather, significant differences between spring and winter canola root system were evident at 40 days after planting and reached maximums at 60 days after planting (Arifuzzaman and Rahman, 2017). In the current study, we

analyzed the transcriptomes to identify differentially expressed genes between spring and winter types canola root system at two different time points, 30 and 60 days after planting. We discussed the differentially regulated genes and gene sets based on their nature, function and involvement in different physiological and hormonal pathways involving root growth and development. To date, this is the first attempt to identify differential gene expression between spring and winter types canola root system to understand the significant variation in the root system architecture between spring and winter canola at later growth stages.

6.3. Materials and methods

6.3.1. Plant materials

Two winter types (Lindore 00 and Regal) and two spring types (Kanada and Wester) germplasms were used in this study. The plants were grown in 40 cm × 10 cm pots in the greenhouse. Sand and peat soil were mixed at an 8:2 ratio and were used as a growing medium. The growing media was supplemented with 10g/pot Osmocote® slow-release fertilizer (Scott's Company LLC, Marysville, OH, USA). Pots were lined with plastic bags, perforated at the bottom before pouring growing media into pot to facilitate root extraction procedures. Perforation allowed the excess water to drain out. Plants were grown in a RCBD with 3 biological replicates in a factorial arrangement with two time points. Thirty and sixty days, were considered as main factor and growth types (spring and winter) were considered as sub factors. Root systems were extracted at 30 days and 60 days after planting and data were taken on Soil level tap root diameter, number of lateral roots, tap root length. Root vigor was scored on a scale described by Arifuzzaman and Rahman, (2017). A small amount of fresh root tissue was collected into liquid nitrogen and stored in -80 ºC freezer. The collected root samples included

taproots, primary root branches and lateral roots. The remaining root samples were dried, and dry weight data was collected for each sample.

6.3.2. RNA extraction, cDNA library preparation and sequencing

RNA were extracted from the root tissue from 22 samples (includes 3 biological replicates for each lines at each time points however libraries failed for two spring type lines at 60 days leaving two biological reps for these lines) following the CTAB extraction method described by Chang et al., (1993). Genomic DNA were removed from the isolated RNA solution by treating with DNAse I (Invitrogen, Carlsbad, CA, USA). RNA quantification and quality were determined by spectroscopy and agarose gel analysis. A total of 22 cDNA libraries were prepared using the NEBnext ultra directional RNA library prep kit according to manufacturer's specifications with minor modifications. mRNA was purified from 5 µg of RNA and first and second strand cDNA were synthesized from the mRNA followed by fragmentation. Fragments between 300 and 400 bases in length were selected using a pipin-prep gel extraction procedure according to manufacturer's protocols, and the quality of the libraries were checked using the Agilent Bioanalyzer DNA7500 DNA chip (Agilent Technologies, Waldbronn, Germany). One hundred base paired end reads were sequenced on Illumina HiSeq2000 platform (Illumina, Inc., San Diego, CA, USA) by BGI America, downloaded into the CyVerse discovery environment, and trimmed for quality (Phred >20) and then size (>70 bases).

6.3.3. Transcriptome analysis

Sequenced data were analyzed by using the Tuxedo suits programs (Trapnell et al., 2012) on CyVerse platform. Paired end reads were aligned to *B. napus* reference genome using Tophat-2.1 in CyVerse discovery environment with an anchor weight of 8 and 0 mismatch. The

aligned reads were then assembled using the cufflink program and fragments per kb per million reads (FPKM) data for individual transcripts were generated. Cuffdiff was used to identify statistically significant genes and transcripts between the samples and time points.

6.3.4. Identifying the key DEGs

The differentially expressed genes (DEGs) in each comparison were considered as the significant if their *p*-value was less than 0.05, and they had a minimum FPKM of 7 in all three biological replicates from at least one treatment group. Different phytohormones have strong direct and indirect effects on plant root development. Complex networks of cross-talk among the phytohormones affecting the root growth are now well established. Consequently, we focused on phytohormone associated genes influencing the different phytohormone signaling pathways, biosynthesis, inactivation and transport. Key DEGs were defined based on their specific functions in root development, their cross-talk with other key phytohormone associated genes and their pattern of differential expressions (higher in spring types or in winter types).

6.3.5. Gene expression pattern

Expression pattern of the significant DEGs were determined based on the log2 transformation of the FPKM fold change ratio at 30 days and 60 days spring vs winter comparison. Fold change was calculated from the ratio of FPKM value in spring types and FPKM value in winter types. Positive value of Log2 (FC) indicates the upregulation of the gene in spring types relative to winter types at respective time point negative value of Log2 (FC) indicates the down regulation of the gene in spring types relative to winter types at respective time point. Finally, expression patterns of genes were determined by its direction of change in spring types at 30 days and 60 days.

6.3.6. Gene set enrichment analyses (GSEA)

Gene Set and Sub-network Enrichment Analysis (GSEA) were done in Pathway Studio 11.4 (Nikitin et al., 2003). GSEA is a useful statistical approach to detect the overrepresented genes, group them as sets and rank those sets based on their FDR *p*-value (Howe et al., 2015). We analyzed the differentially expressed genes those were identified in two comparisons, spring vs winter at 30 days and at 60 days. In the both comparisons, we first identified the gene sets without considering whether they were upregulated either in the spring or winter types. Then we separately identified the gene sets that were upregulated in spring and winter types for individual time point. The parameters were set as described by Howe et al., (2015) except for the FDR *p*-value cut off 0.05 to be considered as significant gene set. Biological process, cellular components and molecular functions were the three Gene Ontology (GO) datasets that we analyzed. *Arabidopsis* annotation information were used to assign the GO terms of *B. napus* genes. For the Pathway Studio dataset, seven types of pathways (expression targets, miRNA targets, protein modification targets, proteins regulating disease, proteins regulating cell processes, binding partners and neighbors of key proteins, and biological processes) were analyzed.

6.4. Results

6.4.1. Phenotypic analysis

At 30 days, no root parameters were found to be statistically significantly different between spring and winter types root system (Fig 6.1). However, four root parameters, root diameter at soil level (R_1Dia), primary root branch (PRB), root vigor score (RVS) and root dry weight (RDW) were significantly greater in the winter types compared to the spring type at 60

days. No significant difference was found between the tap root length of spring and winter types at 60 days.

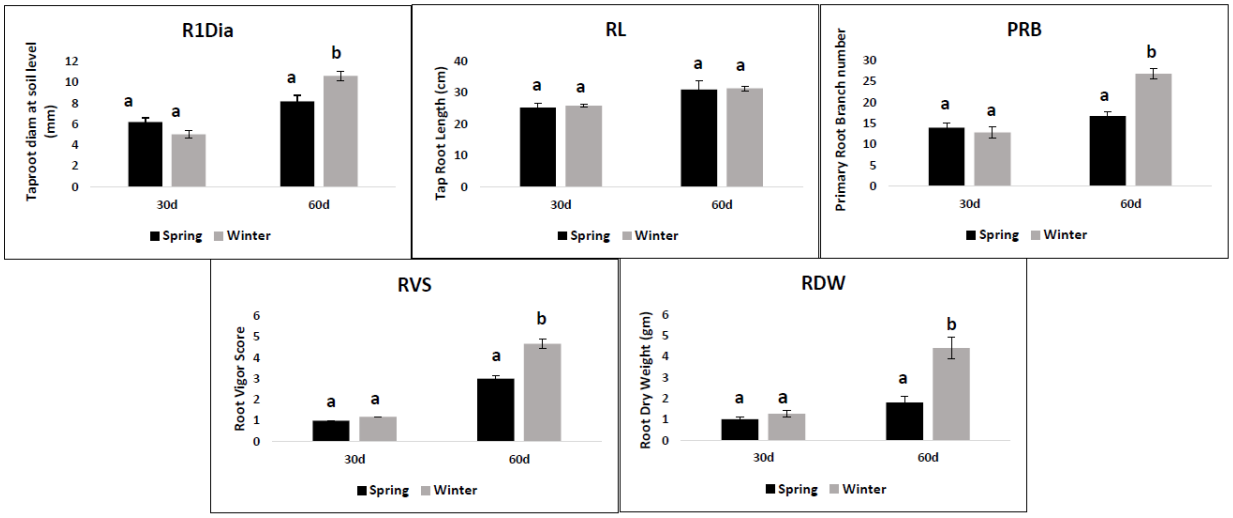


Figure 6.1. Phenotypic observation of different root traits in spring vs winter comparison at 30 days and 60 days

6.4.2. Transcriptome changes between spring and winter types canola

A total 169,646 transcripts were analyzed for differential expression between spring and winter types at two time points (30 days and 60 days) in this study (Fig 6.2b), (Appendix Table A4). Approximately, 10% of the transcripts had no hits with any known *B. napus* gene model. At 30 days after planting, 582 transcripts were found to be significantly differentially expressed (FDR adjusted $p<0.05$) between spring and winter types canola. A relatively lower number, 555 transcripts were significantly differentially expressed between spring and winter types at 60 days after planting. Out of all significant DEGs, 219 genes were differentially expressed between spring and winter types at both time points (Fig 6.2a). These 219 common DEGs showed very similar pattern of expression between spring and winter types. Rest of the significant DEGs were identified as unique to either 30 days (363 significant DEGs) or 60 days (336 significant DEGs). A total of 92 significantly differentially expressed transcripts between spring and winter

types at 30 days could not be aligned with any *Arabidopsis* gene model (Fig 6.2c). In addition, 140 transcripts significantly differentially expressed between spring and winter types at 60 days could not be aligned with any *Arabidopsis* gene model (Fig 6.2d).

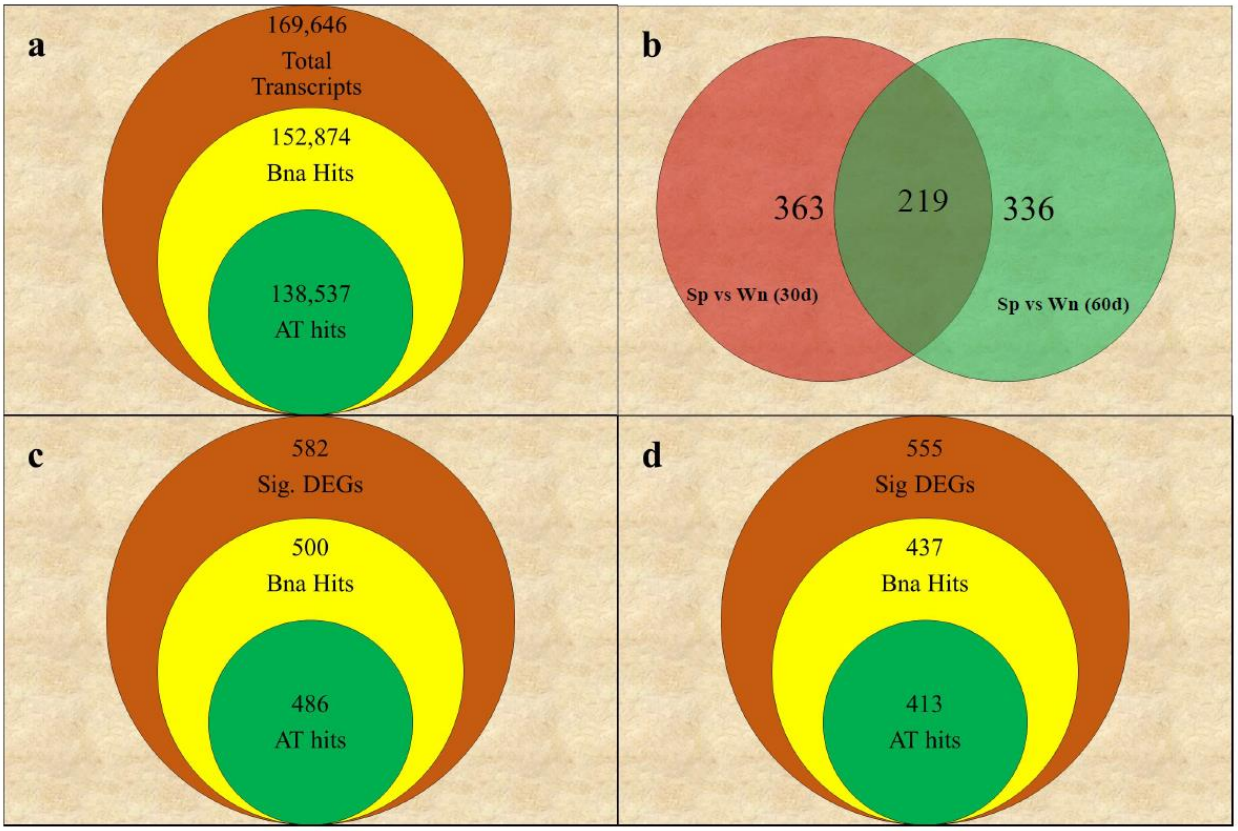


Figure 6.2. Overview of the transcripts. (a) Total number of transcripts that are matched with *B. napus* gene models (Bna) and *Arabidopsis* gene models (AT); (b) Number of significantly differentially expressed genes between spring and winter at 30 and 60 days at both time points; (c) Number of significantly differentially expressed genes between spring and winter at 30 days that matched with BNA and AT gene models; (d) Number of significantly differentially expressed genes between spring and winter at 60 days that matched with BNA and AT gene models.

6.4.3. Gene set enrichment analysis (GSEA) and subnetwork enrichment analysis (SNEA)

GSEA and SNEA were performed for our data set to detect the major gene sets related to key pathways, metabolic processes and regulatory functions responsible for root development in

spring and winter types canola. Significant gene sets were identified using GO terms including biological process, cellular components and molecular functions. For each comparison, spring vs winter at 30 days, and spring vs winter at 60 days, gene sets were estimated based on up-regulated in winter types, up-regulated in spring types, and on significance without considering the direction of upregulation of the genes (Appendix Table A5.1-5.4).

6.4.4. Gene expression pattern

Based on their direction of change in spring types relative to winter types at 30 days and 60 days, significant DEGs were grouped into four possible expression patterns. These are up-regulation at both time points (Pattern 1= Up-Up), up-regulation at 30 days but down-regulation at 60 days (Pattern 2= Up-Down), down-regulation at both time points (pattern 3= Down-Down), down-regulation at 30 days but upregulation in 60 days (Pattern 4= Down-Up). Most of the significant DEGs from both time points followed the either expression pattern 1 or pattern 3 (Appendix Table A6.1-6.3). Only 21 and 14 significant DEGs from 30 days spring vs winter followed expression pattern 2 and pattern 4, respectively. On the other hand, 16 and 14 significant DEGs from 60 days spring vs winter followed expression pattern 2 and pattern 4, respectively. Based on other results and discussion, expression pattern of few selected important significant DEGs from both time points were presented in Fig 6.3.

6.4.5. Differential expression of transcription factor genes

6.4.5.1. Overview

A total of 10,599 transcripts encode transcription factors based on similarity to *Arabidopsis* gene models were identified. Among them, only 49 transcription factors were significantly differentially expressed between spring vs winter at 30 days and 60 days. Among

them, 24 transcription factors were significantly differentially expressed exclusively in spring vs winter at 30 days (Appendix Table A7.1). On the other hand, 15 transcription factors were significantly differentially expressed exclusively in spring vs winter at 60 days (Appendix Table A7.2). The remaining 10 transcription factors were significantly differentially expressed in spring vs winter at both time points (Appendix Table A7.3**).**

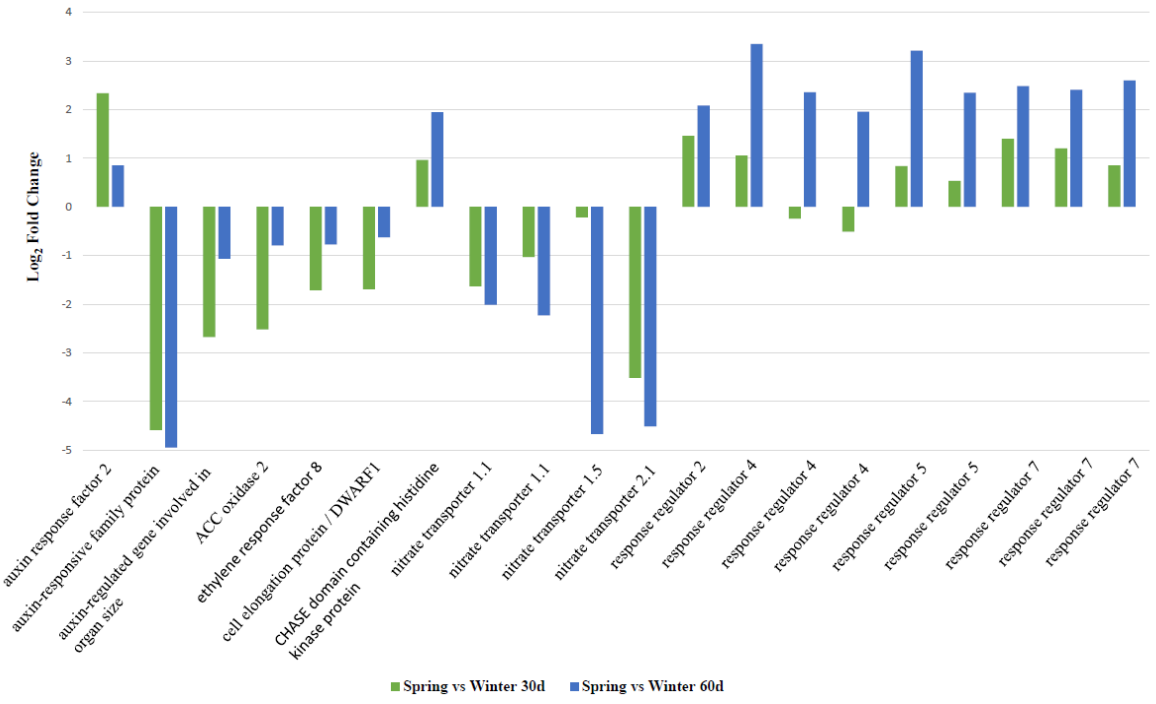


Figure 6. 3. Log2 (FC) of some selected genes in spring vs winter at 30 days and 60 days. Positive FC= Upregulation and Negative FC= Downregulation of the gene in spring types relative to winter types.

6.4.5.2. Transcription factor genes

Top ten significantly differentially expressed transcription factor genes (ranked by FDR *p*-value) for each comparison are presented in Fig 6.4a-b. In spring vs winter at 30 days, relatively higher expressions were observed in winter types for most of the top ten significantly

differentially expressed genes (Fig 6.4a). Some notable genes are BnaCnng54100 encoding proteins similar to "*AUXIN RESPONSE FACTOR 2*", BnaA08g01300 encoding proteins similar to "*ETHYLENE RESPONSE FACTOR 8*", BnaAnng40580 encoding proteins related to "*ABI3/VP1 1*" in *Arabidopsis*. Three transcription factor genes have relatively higher expression in spring types at 30 days comparing to winter type at 30 days. For example, BnaA09g12590 encoding proteins similar to "*ERF family protein 8*", BnaA04g26320 encoding proteins similar to "*AGAMOUS-like 20*" in *Arabidopsis*. Six of the top ten transcription factor genes of spring vs winter at 60 days showed relatively higher expression in winter type (Fig 6.4b). These includes, BnaC02g02200, BnaAnng26200 and BnaA05g27930 genes encoding proteins similar to "*WUSCHEL related homeobox 5*", "NAC domain containing protein 47" and "*DRE/CRT*-binding protein 2B" in *Arabidopsis*. The transcription factor genes showing relatively higher expression in spring type at 60 days comparing to winter type at 60 days includes BnaA06g33560 and BnaC01g22100 encoding proteins similar to "*BASIC LEUCINE-ZIPPER 48*" and "*RESPONSE REGULATOR 2*" in *Arabidopsis*.

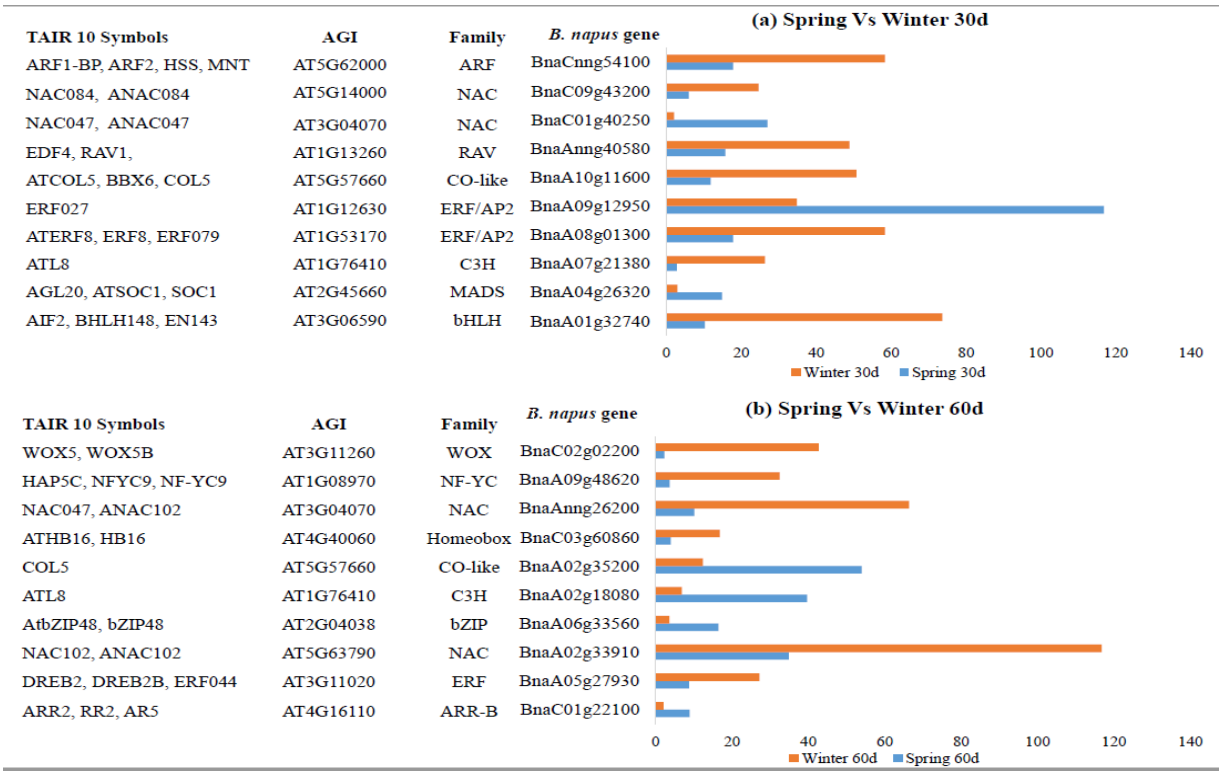


Figure 6.4. Top ten differentially expressed TF genes between spring and winter; (a) 30 days, (b) 60 days.

6.4.5.3. Transcription factor gene sets

A total of 19 transcription factor gene sets were identified as differentially expressed in spring vs winter at 30 days through GSEA and SNEA (Table 6.1). Eight of them were upregulated in winter types (30 days) which includes calmodulin binding, binding partners of *WRKY70* (*WRKY* family transcription factor), binding partners of *BZR1* (*BRASSINAZOLE-RESISTANT 1*), binding partners of *bZIP* transcription factor, upstream neighbors of *WOX5*, upstream neighbors of auxin response factor and upstream neighbors of *DREB1A* (dehydration response element *B1A*). The transcription factor gene sets that were upregulated in spring types (30 days) includes MADS box protein, expression targets of *HSF3* (*HEAT SHOCK FACTOR 3*), expression targets of *HSF*, upstream neighbors of *HSF*, expression targets of *DREB2C* (dehydration response element *B2C*), expression targets of *HSF1*, upstream neighbors of HSF1,

upstream neighbors of *HSFA3*, binding partners of *ABF3* (abscisic acid responsive element-binding factor 3), upstream neighbors of ABF1, upstream neighbors of *WRKY33*, upstream neighbors of *EREBP* (ethylene-responsive element binding factor 13).

A total of 24 transcription factor gene sets were differentially expressed in spring vs winter at 60 days. Ten of them were upregulated in winter types (60 days) including CBF/NF-Y transcription factors, NAC family, *BREB*, expression targets of *BZR2*, expression targets of *HSFA2*, expression targets of *ABI4* (*ABSCISIC ACID INSENSITIVE4*), upstream neighbors of *WRKY*, binding partners of *RGA1* (Repressor of GA1-3 1) etc. The transcription factors that were upregulated in spring types (60 days) includes MADS box protein, expression targets of MYB51 and MYB34, expression targets of *ABI3* and *ABI4*, upstream neighbors of *RR2* and expression target of *RGA1* etc.

Table 6.1. Differentially regulated Transcription Factor gene sets between spring and winter canola at 30 days and 60 days.

Time Point	Upregulation in spring		Upregulation in winter	
	Name	Measured entity	Name	Measured entity
30 days	MADS box protein	8	calmodulin binding	44
	Expression Targets of HSF	11	Binding Partners of WRKY70	5
	Expression Targets of DREB2C	11	Binding Partners of BZR1	14
	Expression Targets of HSF1	9	Binding Partners of bZIP	10
	Binding Partners of ABF3	5	Upstream Neighbors of ARF	11
	Upstream Neighbors of HSF	13	Upstream Neighbors of WOX5	5
	Upstream Neighbors of HSFA3	7	Upstream Neighbors of DREB1A	25
	Upstream Neighbors of HSF1	6	Upstream Neighbors of RRTF1	5
	Upstream Neighbors of ABF1	5		
	Upstream Neighbors of WRKY33	17		
	Upstream Neighbors of EREBP	8		
60 days	MADS box protein	7	CBF/NF-Y transcription factors	9
	Expression Targets of MYB51	6	DREB	5
	Expression Targets of MYB34	9	NAC family	25
	Expression Targets of RGA1	10	Expression Targets of HSFA2	5
	Expression Targets of ABI4	7	Expression Targets of ABI4	8
	Binding Partners of BZR1	16	Expression Targets of HSF	12
	Upstream Neighbors of RGA1	14	Binding Partners of RGA1	9
	Upstream Neighbors of ABF1	5	Upstream Neighbors of ABI4	10
	Upstream Neighbors of ABF2	9	Upstream Neighbors of WRKY	25
	Upstream Neighbors of ABF	6	Upstream Neighbors of WRKY25	7
	Upstream Neighbors of ABI3	13		
	Upstream Neighbors of WOX5	8		
	Upstream Neighbors of HSF	12		
	Upstream Neighbors of RR2	6		

6.4.6. Differential expression of phytohormone related genes

6.4.6.1. Overview

A total of 2,751 transcripts were identified as the phytohormone associated genes involved in putative hormonal function (synthesis, signaling, transport and catabolism) in *Arabidopsis*. Among them, only 10 transcripts were significantly differentially expressed between spring and winter types at 30 days (Appendix Table A8.1). On the other hand, at 60 days, 19 transcripts were differentially expressed between spring and winter types (Appendix Table A8.2). Top ten phytohormone associated significant DEGs at both time points are presented in Fig 6.5a-b. With GSEA and SNEA, 22 gene sets were identified as phytohormone associated gene sets in spring vs winter at 30 days (Table 6.2). Three of them were upregulated in both spring and winter types. In spring vs winter comparison at 60 days, thirty-two phytohormone associated gene sets were differentially expressed (Table 6.2). Among them, only eight gene sets were found to be upregulated in winter types, whereas twenty gene sets were upregulated in spring types.

6.4.6.2. Auxin associated genes and gene sets

All the significantly differentially expressed auxin associated genes in spring vs winter at 30 days showed similar pattern of relatively higher expression in spring types (Appendix Table A8.1). Three of them are BnaA10g14600, BnaCnng54100 and BnaA01g24190 encoding proteins similar to *AAO1* (*ALDEHYDE OXIDASE 1*), *ARF2 (AUXIN RESPONSE FACTOR 2), AUX/IAA* (*INDOLE-3-ACETIC ACID INDUCIBLE 2)* in *Arabidopsis*. The other auxin associated gene, BnaCnng62240 encoding protein similar to *CYP71A13* (cytochrome P450, family 71, subfamily A, polypeptide 13) in *Arabidopsis* significantly differentially expressed in spring vs winter at both 30 days and 60 days. Five more auxin associated transcripts significantly differentially

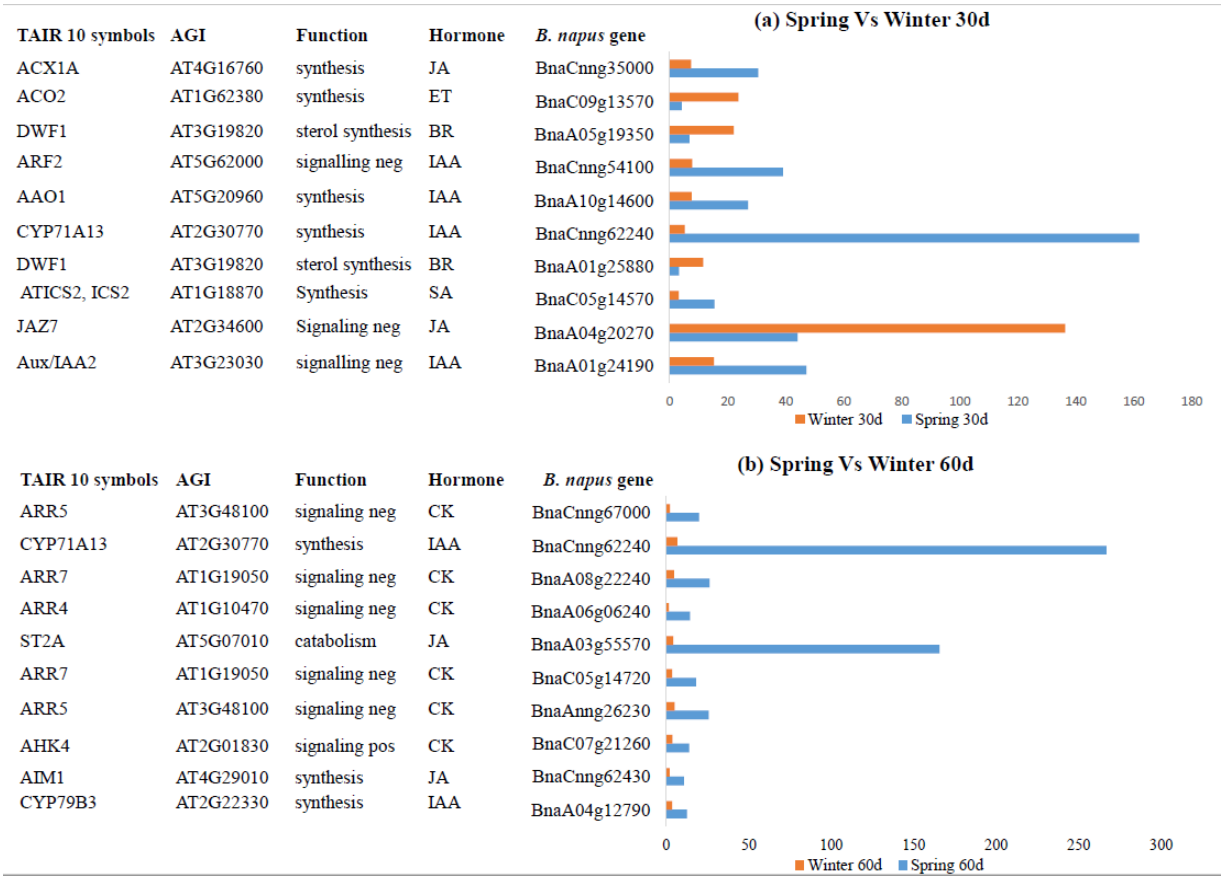
expressed between spring and winter types at 60 days encoding three proteins similar to *Arabidopsis*- C*YP79B3, CYP79B2* and *NRT1.1 (NITRATE TRANSPORTER 1.1*) (Appendix Table A8.1). Among them, only *NRT1.1* showed relatively higher expression in winter types than the spring types.

Interestingly, at 30 days of spring vs winter, 10 auxin associated gene sets were upregulated in winter types. Some of them are related to auxin responsive family proteins (*IAA*s, upstream neighbors of *IAA7*, upstream neighbors of *IAA5*, and upstream neighbors of *IAA*s). Others are response to auxin, auxin-activated signaling pathway, binding partners of *TIR1* (*TRANSPORT INHIBITOR RESPONSE 1*), upstream neighbors of *ARF*. Three gene sets IAAs, auxin-activated signaling pathway and binding partners of *TIR1* were also upregulated in spring types. The other two auxin associated gene sets (plant growth auxin signaling and senescence auxin signaling) related to plant growth and senescence were also upregulated in spring types. At 60 days spring vs winter, only three gene sets were differentially regulated. Two of them, basipetal auxin transport and upstream neighbors of *AXR1* (auxin resistance 1) were upregulated in winter types and binding partners of *TIR1* was upregulated in spring types.

6.4.6.3. Cytokinin (CK) associated genes and gene sets

No significantly differentially genes associated with CK were identified at 30 days spring vs winter. However, at 60 days spring vs winter, 10 transcripts encoding genes associated with CK in *Arabidopsis* were found to be significantly differentially expressed (Appendix Table A8.1-A8.2). These 10 transcripts encode Type-A Response Regulators (*RR4, RR5* and *RR7*), Type-B Response Regulator (*RR2*) and *ARABIDOPSIS HISTIDINE KINASE 4 (AHK4).* Interestingly, all these CK associated genes showed similar pattern of higher relative expression in spring types (60 days) comparing to winter types (60 days). We found very similar results in

GSEA and SNEA data. No gene sets associated with CK were detected at 30 days spring vs winter. On the other hand, seven differentially regulated gene sets were identified as CK associated at 60 days spring vs winter. These are *ARR*-A type family, Cytokinin-activated signaling pathway, cytokinin signaling, response to cytokinin, upstream neighbors of *RR22*, upstream neighbors of *RR24* and upstream neighbors of *RR6*. All of these CK associated gene sets were upregulated in spring types (60 days). When filtered by significance level, two CK associated gene sets, cytokinin activated signaling pathway and response to cytokinin stimulus were found to be upregulated in spring types (60 days).



TAIR 10 symbols	AGI	Function	Hormone	*B. napus* gene
ACX1A	AT4G16760	synthesis	JA	BnaCnng35000
ACO2	AT1G62380	synthesis	ET	BnaC09g13570
DWF1	AT3G19820	sterol synthesis	BR	BnaA05g19350
ARF2	AT5G62000	signalling neg	IAA	BnaCnng54100
AAO1	AT5G20960	synthesis	IAA	BnaA10g14600
CYP71A13	AT2G30770	synthesis	IAA	BnaCnng62240
DWF1	AT3G19820	sterol synthesis	BR	BnaA01g25880
ATICS2, ICS2	AT1G18870	Synthesis	SA	BnaC05g14570
JAZ7	AT2G34600	Signaling neg	JA	BnaA04g20270
Aux/IAA2	AT3G23030	signalling neg	IAA	BnaA01g24190

TAIR 10 symbols	AGI	Function	Hormone	*B. napus* gene
ARR5	AT3G48100	signaling neg	CK	BnaCnng67000
CYP71A13	AT2G30770	synthesis	IAA	BnaCnng62240
ARR7	AT1G19050	signaling neg	CK	BnaA08g22240
ARR4	AT1G10470	signaling neg	CK	BnaA06g06240
ST2A	AT5G07010	catabolism	JA	BnaA03g55570
ARR7	AT1G19050	signaling neg	CK	BnaC05g14720
ARR5	AT3G48100	signaling neg	CK	BnaAnng26230
AHK4	AT2G01830	signaling pos	CK	BnaC07g21260
AIM1	AT4G29010	synthesis	JA	BnaCnng62430
CYP79B3	AT2G22330	synthesis	IAA	BnaA04g12790

Figure 6.5. Top ten differentially expressed phytohormone associated genes between spring and winter; (a) 30 days, (b) 60 days.

6.4.6.4. Abscisic acid (ABA) associated genes and gene sets

No significantly differentially expressed gene associated with abscisic acid were identified in either of the comparison, 30 days spring vs winter, and 60 days spring vs winter. However, two abscisic acid associated gene sets were detected at 30 days spring vs winter and found to be upregulated in spring types (30 days). These are binding partners of *ABF3* (ABA response element binding factor 3) and upstream neighbors of *ABF1*. Several ABA associated gene sets were detected at 60 days spring vs winter. Five of them were upregulated in winter types (60 days) including abscisic acid binding, abscisic acid receptor, expression targets of *ABI4* (*ABA INSENSITIVE 4),* upstream neighbors of *ABI4* and upstream neighbors of *ABI1*. Six of them were upregulated in spring types (60 days) which included response to abscisic acid, binding partners of *ABI2*, upstream neighbors of *ABF1*, upstream neighbors of *ABF2*, upstream neighbors of *ABF*, and upstream neighbors of *ABI3*.

6.4.6.5. Gibberellin (GA) associated genes and gene sets

No GA associated genes were found to be significantly differentially expressed between spring vs winter comparison at either of the time points, 30 days and 60 days. Few differentially regulated gene sets were identified through GSEA and SNEA. At 30 days spring vs winter, one GA associated gene set, upstream neighbors of *GA20OX1* (*GIBBERELLIN 20 OXIDASE 1*) were found to be upregulated in winter types (30 days). One gene set, binding partners of *RGA1* were upregulated in winter types at 60 days. In contrast, seven differentially regulated GA associated gene sets were found to be upregulated in spring types in 60 days in spring vs winter comparison. These are, gibberellic acid mediated signaling pathway, gibberellin signaling, response to gibberellin, binding partners of *GAI (GIBBRELLIC ACID INSENSITIVE),* upstream neighbors of

RGA1 (*Repressor of GA 1*), expression target of *RGA1*, binding partners of *RGA1* and upstream neighbors of *SLY1* (*SLEEPY 1*).

Table 6.2. Differentially regulated phytohormone associated gene sets between spring and winter types canola at 30 days and 60 days.

Time Point	Upregulated in spring			Upregulated in winter		
	Name	Measured entity	Phytohormone	Name	Measured entity	Phytohormone
30 days	Binding Partners of ABF3	5	ABA	response to brassinosteroid	7	BR
	Upstream Neighbors of ABF1	5	ABA	Binding Partners of BZR1	14	BR
	response to brassinosteroid	9	BR	Binding Partners of BRI1	6	BR
	Plant Growth Auxin Signaling	15	IAA	Upstream Neighbors of GA20OX1	10	GA
	IAAs	11	IAA	response to auxin	67	IAA
	Senescence Auxin Signaling	16	IAA	auxin-activated signaling pathway	36	IAA
	auxin-activated signaling pathway	39	IAA	IAAs	7	IAA
	Binding Partners of TIR1	6	IAA	Binding Partners of SCF(TIR1) complex	5	IAA
	response to salicylic acid	48	SA	Binding Partners of TIR1	9	IAA
				Upstream Neighbors of ARF	11	IAA
				Upstream Neighbors of IAA7	6	IAA
				Upstream Neighbors of IAA5	5	IAA
				Upstream Neighbors of IAAs	15	IAA
				Upstream Neighbors of AXR1	9	IAA
				response to jasmonic acid	45	JA
				JA, Et, and SA Crosstalk Signaling	18	JA, ET, SA
60 days	Binding Partners of ABI2	5	ABA	abscisic acid binding	5	ABA
	Upstream Neighbors of ABF1	5	ABA	abscisic acid receptor	5	ABA
	Upstream Neighbors of ABF2	9	ABA	Expression Targets of ABI4	8	ABA
	Upstream Neighbors of ABF	6	ABA	Upstream Neighbors of ABI4	10	ABA
	Upstream Neighbors of ABI3	13	ABA	Upstream Neighbors of ABI1	16	ABA
	Binding Partners of BZR1	16	BR	Binding Partners of RGA1	9	GA
	ARR-A type family cytokinin-activated signaling pathway	5 17	CK CK	basipetal auxin transport Upstream Neighbors of AXR1	5 6	IAA IAA

Table 6.2. Differentially regulated phytohormone associated gene sets between spring and winter types canola at 30 days and 60 days (Continued).

Time Point	Upregulated in spring			Upregulated in winter		
	Name	Measured entity	Phytohormone	Name	Measured entity	Phytohormone
	Upstream Neighbors of RR24	5	CK			
	Upstream Neighbors of ARR6	8	CK			
	Upstream Neighbors of RR2	13	CK			
	cellular response to ethylene stimulus	5	ET			
	GA mediated signaling pathway	11	GA			
	Gibberellin Signaling	13	GA			
	Expression Targets of RGA1	10	GA			
	Binding Partners of GAI	8	GA			
	Upstream Neighbors of RGA1	14	GA			
	Upstream Neighbors of SLY1	9	GA			
	tryptophan biosynthetic process	10	IAA			
	Upstream Neighbors of TIR1	5	IAA			
	Upstream Neighbors of CYP79B3	9	IAA			
	Upstream Neighbors of CYP83B1	6	IAA			

6.4.6.6. Brassinosteroid (BR) associated genes and gene sets

Two significantly differentially expressed transcripts encoding BR associated gene in *Arabidopsis* at 30 days spring vs winter were identified. Both of these transcripts encode proteins similar to *DWF1 (DWARF1)* in *Arabidopsis*. However, their pattern of expression is different from each other, one had higher relative expression in spring types (30 days), and the other had higher relative expression in winter types (30 days). Two BR associated gene sets were identified as differentially regulated in 30 days spring vs winter. One of them, response to brassinosteroids, were found to be upregulated in both winter and spring types (30 days) whereas the other one, binding partners of *BRI1* (*BRASSINESTOROIDS INSENSITIVE 1*) was upregulated only in winter types (30 days). No BR associated gene or gene sets were detected in 60 days spring vs winter comparison.

6.4.6.7. Ethylene (ET) associated gene and gene sets

In 30 days spring vs winter, *ACO2* (*ACC oxidase 2*) involve in ET signaling was found to be significantly differentially expressed and had higher relative expression in winter types (30 days). One ET enriched gene set, cellular response to ethylene stimulus, were found to be differentially regulated in 60 days spring vs winter comparison where it was upregulated in spring types (60 days).

6.4.6.8. Jasmonic acid (JA) associated genes and gene sets

Two transcripts encoding proteins similar to *ST2A (SULFOTRANSFERASE 2A*) and AIM1 *(ABNORMAL INFLORESCENCE MERISTEM)* genes associated with JA were found to be significantly differentially expressed in 60 days spring vs winter. Both of them showed higher relative expression in spring types (60 days). One JA associated gene set, response to jasmonic

acid, was found to be differentially regulated in spring vs winter at both 30 days and 60 days. It was upregulated in spring types in 30 days spring vs winter whereas, it was upregulated in winter types in 60 days in spring vs winter comparison.

6.4.6.9. Salicylic acid (SA) associated genes and gene sets

Two different transcripts encoding proteins similar to SA associated genes in *Arabidopsis* were identified in spring vs winter at each of the time points, 30 days and 60 days. These are *ISC2* (*ISOCHORISMATE SYNTHASE 2*) in 30 days spring vs winter and *MES2 (METHYLESTERASE 2)* in 60 days spring vs winter. Both of these genes had relatively higher expression in spring types. One gene set, response to salicylic acid, was found to be upregulated in spring types in 30 days in spring vs winter comparison.

6.5. Discussion

6.5.1. Transcription factor genes and gene sets

6.5.1.1. Abundance of transcription factor genes from ERF, NAC and bHLH family

ERF family transcription factors are involved in plant growth and development, different phytohormone signaling and different biotic and abiotic stress responses (Nakano et al., 2006; Xu et al., 2011). It has been reported that overexpression of ERF transcription factor genes can improve drought, salt and freezing tolerances in transgenic plants (Xu et al., 2011). Although nine ERF family transcription factor genes were significantly differentially regulated between spring vs winter at both time points, they did not have an expression pattern indicative of an obvious role in differentiating between winter or spring root developmental responses.

In total, six transcripts encoding four different NAC transcription factor genes were differentially expressed between spring and winter types at 30 days and 60 days. Among them, *NAC102* and *NAC047* transcription factor genes which express under hypoxia and waterlogging stress (Christianson et al., 2009; Rauf et al., 2013) were significantly differentially expressed at both time points. Six bHLH transcription factor genes were differentially expressed between spring and winter in the current study, did not follow any specific pattern of relative expression.

6.5.1.2. Other transcription factor genes

ARF2 transcription factor (significant at 30 days) promotes flowering and stamen development, flower abscission and leaf senescence (Ellis et al., 2005; Okushima et al., 2005) with relative higher expression in spring types and expression pattern "Up-Up". This is expected because only spring type flowers without vernalization in the current experiment. However, it was surprising to find this correlation in root tissues. A root related transcription factor gene, *WOX 5* was differentially expressed at 30 days with relative higher expression in winter types and expression pattern "Down-Down".*WOX5* usually expresses in root apical meristem and maintain root quiescent center (QC) which is important for root stem cell niche and root growth (Forzani et al., 2014; Kong et al., 2015; Lopez-Moya et al., 2017).

6.5.1.3. Transcription factor gene sets

In single gene analysis, we usually evaluate the statistically significant DEGs and may overlook a vast number of non-significant DEGs sharing common functions and lead to a common expression pattern (Howe et al., 2015). GSEA is a very useful tool to estimate the expression of gene sets comprising of large number of genes with similar biological pathways and regulatory functions within the DEGs list (Subramanian et al., 2005; Howe et al., 2015)

The transcription factors gene sets that were found to be differentially regulated between spring and winter at 30 days and 60 days are mostly associated with different abiotic stresses. At 30 days spring vs winter, gene sets related to heat shock factor (*HSF*) transcription factor family were most abundant among the differentially expressed gene sets and interestingly, they all were upregulated in spring types. *HSF*s usually play a major roles in plant to abiotic stresses tolerance by binding with the *HSE*-cis element of promoter of the stress responsive gene (Guo et al., 2016). However, at 60 days spring vs winter, *HSF* related gene sets were upregulated in both spring and winter types relative to 30 days samples. Another notable stress related gene set "expression target of *DREB2C*" were found to be upregulated in spring type relative to winter type at 30 days. *DREB2C* is a member of ERF transcription family and overexpression of *DREB2C* can induce *HSFA3* transcription factor in both high temperature stress and non-stress condition (Chen et al., 2010; Ohama et al., 2016)

At 60 days, *CBF/NFY*, *DREB* (member of ERF) and NAC transcription factor gene sets were upregulated only in winter types. Upstream neighbor of *RR2* was found to be upregulated in spring types at 60 days relative to winter type. RR2 is a type-B ARR transcription factor and overexpression of *ARR2* or *RR2* caused increased sensitivity of cytokinin (Hwang and Sheen, 2001).

6.5.2. Significantly differentially expressed phytohormone genes and gene sets

6.5.2.1. Auxin associated genes and gene sets

Auxin or indole-3-acetic acid (IAA) is one of the major phytohormones which plays significant roles in all stages of plant growth including root growth and development (Ludwig-Müller, 2011). Auxin biosynthesis in shoot and root, its transportation (short and long distance) and signaling are the major steps of auxin mediated root growth and development (Saini et al.,

2013). In the current study, all the auxin associated significant DEGs either in 30 days or 60 days spring vs winter comparison have shown relative higher expression in spring types except *NRT1.1*. Significantly differentially expressed *AAO1*, *ARF2* and *AUX/IAA2* genes are involved in auxin biosynthesis and signaling process. *AAO1* gene converts indole-3-acetaldehyde (IAAld) into IAA in tryptophan (Trp) dependent pathway of auxin biosynthesis (Seo et al., 1998). *AUX/IAA* gene family plays a negative role in auxin signaling process by inhibiting *ARF* transcription factors to activate auxin responsive genes (Szemenyei et al., 2008). Notably, *ARF2* is induced by ABA and can inhibit the negative effect of ABA in cell division and auxin distribution when overexpressed (Wang et al., 2011a). *CYP79B2* and *CYP79B3* encoding cytochrome P450s are involved in converting Trp to indole-3-acetaldoxime (*IAOX*), a precursor of IAA in Trp dependent auxin biosynthesis pathway (Zhao et al., 2002). However, the mix of auxin response-promoting and limiting genes did not provide a clear indication as to the impact of auxin in the differences observed between spring and winter types root growth morphology.

In GSEA and SNEA, auxin associated gene sets were the most abundant among the differentially regulated phytohormone associated gene sets at 30 days. The majority of these differentially regulated auxin associated gene sets were found to be upregulated in winter types. This is in contradiction with the single gene analysis where we observed all the auxin associated genes have higher relative expression in spring types. The auxin gene sets upregulated in winter types at 30 days were mostly related to *Aux/IAA* gene family which, with several exceptions, is a negative regulator of *ARF* transcription factor family (Szemenyei et al., 2008). At 60 days spring vs winter only three gene sets were differentially regulated and none of them were related to *Aux/IAA* gene family. One gene set, "binding partners of *TIR1*" is upregulated in both spring and winter types at 30 days relative to 60 days, and in spring types at 60 days relative to winter type

at 60 days. With high auxin concentration *Aux/IAA* binds with *TIR1* instead of *ARF*s and this leads to transcriptional activation of auxin responsive genes by ARFs (Gray et al., 2001; Ramos et al., 2001; Zenser et al., 2001; Mockaitis and Estelle, 2008; Lau et al., 2009).

To summarize, auxin associated genes related to auxin biosynthesis were identified in single gene analysis in both 30 days and 60 days spring vs winter comparison with a relative higher expression in spring types. In GSEA and SNEA, majority of the differentially regulated gene sets were identified in 30 days spring vs winter comparison and most of them are related to *Aux/IAA* gene family with an upregulation in winter types.

6.5.2.2. Cytokinin associated genes and gene sets

Unlike auxin, cytokinin associated significantly differentially expressed genes and gene sets showed specific and distinct pattern of expression with a relative higher expression of spring type, only in 60 days spring vs winter. Interestingly, all these cytokinin associated DEGs and gene sets were mostly related to *ARR* or *RR*. In *Arabidopsis*, there are two types of *RR* genes, type-A *RR* and type-B *RR*. Both types are highly induced by cytokinin through a multistep phosphorelay system involving hybrid type *AHK*, histidine phosphotransfer protein (*AHP*s) and response regulators (To et al., 2004; Ferreira and Kieber, 2005). Cytokinin is perceived by *AHK*s through *CHASE* (cyclases/ histidine kinases associated sensory extracellular) domain in plasma membrane, and then through multistep phosphorelay the phosphoryl group is transferred to the receiver domain of type-B *ARR*s. Type-B *ARR*s act as the transcription factors and activate the type-A *ARR*s and other cytokinin response genes (Ferreira and Kieber, 2005). In this current study, type-A (*ARR4*, *ARR5* and *ARR7*), type-B *ARR*s (*ARR*2) and a chase domain containing *AHK4* were upregulated in 60 days with a relatively higher expression in spring types. This evidence suggests that higher concentrations of cytokinin was present in the root system of

spring type canola at 60 days. Further tests would be needed to determine if this is indeed the case or if cytokinin signaling was enhanced in spring types via other mechanisms.

Several studies described the negative regulatory role of cytokinin in root growth and development (Riefler, 2006; Dello Ioio et al., 2008). Targeted expression of *ISOPENTENYL TRANSFERASE* (*IPT*), a cytokinin biosynthesis in the xylem pole pericycle cells resulted defective lateral root initiation and patterning (Laplaze et al., 2007). In another study, Dello Ioio et al. (Dello Ioio et al., 2008) showed that, cytokinin responsive ARR1 (type-B) transcription factor decrease the root meristem size by negatively regulating the auxin efflux carrier PIN through the activation of *short hypocotyl 2* (SHY2) gene. However, with the increased auxin concentration, SHY2 protein is degraded and *PIN* activity is restored. This suggests the role of auxin-cytokinin cross-talk is important in root growth and development. Type-A *ARR*s are regarded as the negative regulator of cytokinin signaling (To et al., 2004; Argyros et al., 2008). Loss of function mutant of eight type-A *ARR* (*ARR* 3, 4, 5, 6, 7. 8. 9, 15) also caused inhibition of auxin transport by altering PIN proteins which ultimately results reduced root apical meristem (To et al., 2007).

To summarize, higher expression of both type-A and type-B *ARR*s and *AHK4* in 60 days spring types root system indicates cytokinin mediated reduced root growth in spring types canola comparing to winter canola at 60 days. Our GSEA and SNEA results with six differentially regulated cytokinin associated gene sets also support this hypothesis. All these cytokinin associated gene sets were differentially regulated in only 60 days spring vs winter comparison with an upregulation in spring types. In addition to type-A and type-B, two gene sets related to type-C response regulators (*RR22* and *RR24*) named “upstream neighbor of *RR22*” and “upstream neighbor of *RR22*” were differentially regulated with a higher expression in spring

types. *ARR22* inhibits the phosphorylation of type-B *ARR*s, therefore the transcription of type-A *ARR*s and other cytokinin responsive genes (Wallmeroth et al., 2017).

6.5.2.3. Abscisic acid associated genes and gene sets

Abscisic acid is a major phytohormone involved in plant growth and development and plays a critical role in different abiotic stress responses in crop (Choi et al., 2000; Tuteja, 2007; Danquah et al., 2014). Plants maintain its cellular ABA balance by continuous biosynthesis and degradation of ABA (Tuteja, 2007). Different abiotic stresses largely disturb the cellular ABA balance by influencing the ABA biosynthesis and degradation (Cutler and Krochko, 1999). ABA signaling pathway is regarded as one of the major regulators of different stress related gene expression and provide adaptation and/or tolerance to various abiotic stresses such as salinity, drought and light (Verslues et al., 2006; Tuteja, 2007). In the current study, significantly more ABA associated gene sets were differentially regulated in 60 days spring vs winter compared to 30 days spring vs winter. Among the ABA associated gene sets, two important components of ABA signaling pathway, *ABI*s and *ABF*s are the most abundant. Some of the *ABI*s (*ABI1* and *ABI2*) act as negative regulator in the ABA signaling pathway (Saez et al., 2004; Hirayama and Shinozaki, 2007). On the other hand, *ABF*s are transcription factor genes, which transcriptionally activate ABA responsive genes. In the presence of ABA, *ABI1* and *ABI2* become inactivated through binding with ABA receptor which leads to the activation of ABF transcription factors facilitating the transcription of ABA responsive genes (Danquah et al., 2014). Three gene sets associated with *ABI3* and *ABI4* were also differentially regulated in 60 days spring vs winter. *ABI3* and *ABI4* are transcription factors and can activate ABA responsive genes (Brady et al., 2003). Large number of differentially regulated ABA associated gene sets at 60 days spring vs

winter suggests that plants faced different abiotic stresses during its growth and development at later growth stages.

6.5.2.4. Gibberellin associated genes and gene sets

Gibberellin is an important class of plant hormone involved root and stem elongation, leaf expansion, flower and seed development, seed germination and maintaining root meristem size in plants (Yamaguchi, 2008; Ubeda-Tomás et al., 2009). Evidence suggests that GA biosynthesis occurs in root meristem and is then transported and accumulated in root epidermis cells in root elongation zone where GA promotes cell elongation and this phenomenon plays a positive role in root elongation (Ubeda-Tomás et al., 2009; Shani et al., 2013). In contrast to promoting root elongation, several studies suggested GA is involved in inhibiting lateral root formation in *Populas*. GA biosynthesis mutant in tomato and GA signaling mutant *Populas* lines produced higher lateral root formation than wild types (Berova and Zlatev, 2000; Busov et al., 2006). Exogenous application of GA can prevent lateral root formation through inhibiting the initiation of lateral root primordia (Gou et al., 2010). In *Populas*, GA deficient and GA insensitive mutants shows higher number of lateral root primordia leading to lateral root development (Gou et al., 2010). Authors concluded that *Populas* root specific auxin efflux career *PIN9* gene is highly GA responsive and upregulated in GA deficient and mutant plants and may play an important role in polar auxin transport to promote lateral root growth in *Populas*.

In the current study, six GA signaling associated gene sets were upregulated in spring types at 60 days, indicates GA mediated root elongation and reduced lateral root growth in spring types at 60 days, which is in accordance with our phenotypic observation. However, three of these gene sets are related to *DELLA* proteins- *GAI* and *RGA1* which are considered as negative regulators of GA response. *DELLA* proteins bind with GA and *GID1* (*GA-Insensitive Dwarf 1*)

two compound complex and form *GA-GID1-DELLA* three compound complex to inhibit GA response (Willige et al., 2007; Pacifici et al., 2015). On the contrary, *DELLA* protein mediated negative GA response can be overruled by a positive regulator of GA response gene named *SLY1* (*SLEEPY1*) which binds with *DELLA* proteins and degrade them to trigger the GA response (Fu, 2004). Interestingly, we observed a *SLY1* associated gene set which is upregulated in spring type at 60 days and thus may suggest a role for high GA response in spring types at 60 days. Therefore, although half of the total GA signaling associated gene sets were upregulated in spring types at 60 days, they are negative regulators of GA response Thus, *SLY1* mediated recovery of GA response and activity might possibly be a mechanism regulating differences observed between spring and winter canola root growth habits.

6.5.2.5. Brassinosteroid associated genes and gene sets

BRs positively regulate root growth and development mainly through root cell elongation and BR deficient or mutant plants show reduced root phenotype (Fridman et al., 2014; Wei and Li, 2016). Beside this, BR also modulate root growth and development through controlling root meristem size, root hair formation, lateral root growth (reviewed by Wei and Li, (2016)). The differentially regulated BR genes and gene sets in the current study were related to both BR biosynthesis (*DWF1*) and BR signaling (*BRI1*, *BZR1* and *BES1*) and were upregulated in both spring and winter types. *DWF1*, *DWF4* and *constitutive photomorphogenesis and dwarf* (*CPD*) are the major BR biosynthesis genes in plant. Synthesized BR binds with BRI1 receptor resulting stimulation of *BRI1*. Stimulated *BRI1* then bind with *BAK1* forming *BRI1-BAK1* complex which is responsible for degradation of *BIN2*, a negative regulator of BR signaling. Degradation of *BIN2* causes activation of transcription factors *DES1* and *BZR1* which transcriptionally activate the BR biosynthesis genes *(DWF4, CPD*) (reviewed by Saini et al., (2013)).

6.5.2.6. Ethylene associated genes and gene sets

Ethylene negatively regulate root growth by inhibiting root cell elongation (Le et al., 2001), cell proliferation in root meristem (Street et al., 2015) and lateral root formation (Negi et al., 2008). Upregulation of gene set "cellular response to ethylene stimulus" in spring types at 60 days spring vs winter indicates ethylene mediated root growth inhibition in spring type. But it is hard to conclude if ethylene has a role in root growth difference between spring and winter types at 60 days with upregulation of just one gene set. Moreover, ethylene mediated root growth inhibition involves cross-talk with other major phytohormones like auxin (Negi et al., 2009; Street et al., 2015) which leads to a more complex system of how ethylene inhibit the root growth and development.

6.5.2.7. Jasmonic and Salicylic acid associated genes and gene sets

Few JA and SA associated genes and gene sets were differentially regulated between spring vs winter at both time points. However, they did not follow any specific pattern or direction of upregulation or downregulation. Both JA and SA are involved in plant defense responses subjected to necrotrophic pathogen (JA), herbivorous insects (JA) and biotrophic pathogen (SA) (Caarls et al., 2015). In addition to the defense response they cross-talk with other phytohormones and play important roles including participating in abiotic stress signaling and plant growth and development (Pieterse et al., 2012). JA associated *AIM1* transcription factor which is a significant DEG is 60 days spring vs winter is involved in mediating cross-talk between biotic and abiotic stress responses (Abuqamar et al., 2009).

6.5.3. Other differentially regulated genes and gene sets

6.5.3.1. Differentially expressed nitrate transporter gene

Nitrogen is one of the essential plant nutrients playing major role in plant growth and development. Plant acquire N from the soil through its root system in form of nitrate and ammonia (Kiba and Krapp, 2016). There are two major nitrogen transport gene families, NRT1 and NRT2 paly key role in plant nitrogen acquisition in different plants (Nacry et al., 2013; Kiba and Krapp, 2016). In our study, we observed higher relative expression of two genes from NRT1 and one gene from NRT2 family in winter type comparing to spring type at 60d. NRT genes contribute in shaping root system architecture in a complex manner and depends mainly on the soil N concentration and subsequent cross talk with different phytohormones.

NRT1.1 is the only dual affinity gene which is upregulated under both high and low nitrate concentration of the soil and inhibit lateral growth formation by promoting basipetal auxin transport out of lateral roots (Krouk et al., 2010). This is in contradiction with the current study as we observed upregulation of two transcripts encoding NRT1.1 in winter type relative to spring type at 60d but instead of inhibition, primary root branches were significantly higher in winter types than spring types at 60d. Interestingly, some studies showed that plants produced increased lateral root formation and elongation when faced local high nitrate concentration under heterogeneous soil nitrate application (Drew and Saker, 1975; Zhang et al., 1999; Yu et al., 2014; Huang et al., 2015). This is due to the role play of nitrate as a signal rather than nutrient in regulating lateral root formation and elongation in a nitrate rich patch (Zhang et al., 1999; Remans et al., 2006; Krouk et al., 2010; Sun et al., 2017). Under local high nitrate condition, NRT1.1 can upregulate the ANR1 transcription factor gene which is a positive regulator of nitrate stimulated LR development (Zhang and Forde, 1998; Zhang et al., 1999; Remans et al.,

2006; Gan et al., 2012; Sun et al., 2017). In contrast to NRT1.1, other NRT1 family genes and NRT2 family genes are upregulated with mild nitrate deficiency and increase the lateral root initiation, formation and elongation. Under low nitrate supply NRT2.1 gene is upregulated resulting the upregulation of auxin biosynthetic gene TAR2. In another pathway, under mild nitrate deprivation, NRT2.1 can upregulate MADs box gene Agamous like-21 (AGL21) resulting upregulation of auxin biosynthetic gene like TAR3. Upregulation NRT2.1 mediated auxin biosynthetic genes TAR2, TAR3 increase the level of auxin in developing lateral roots and promote lateral formation and elongation (reviewed by Sun et al., 2017). Another study in rice showed, NRT2.1 can increase expression of PINs in roots to positively regulate polar auxin transport in lateral root primordia and promote lateral root initiation (Huang et al., 2015).

In addition to their roles in regulating lateral roots, both NRT1 and NRT2 family genes are involved in nitrate uptake from the soil (Nacry et al., 2013; Krapp et al., 2014). Upregulation of these genes in winter type at 60d may result higher nitrate acquisition by winter type canola plants in later growth stage. NRT1.5 is solely a nitrate transporter gene which expressed in root pericycle cells close to xylem and involved in nitrate loading in the xylem to transport to shoot (Lin et al., 2008; Chen et al., 2012). Higher expression of NRT1.1, NRT1.5 and NRT2.1 in winter type root system at 60d indicates higher nitrate uptake and translocation from root to shoot in winter type canola which may be a contributing factor to the usual higher vegetative growth in winter type comparing to spring type canola.

One possible reason for the observed relative lower expression of NRT2.1 gene is spring type at 60d might be related to cytokinin. As discussed earlier, higher concentration of cytokinin might be present in spring type roots at 60d based on the evidence of observed higher relative expression of cytokinin associated gens and gene sets in spring type comparing to winter type at

60d. Cytokinin is proved to be a regulator of both local and systemic signals coordinating N demand and acquisition (Kiba and Krapp, 2016). Cytokinin may repress NRT2.1 gene under N-deprived soil by producing N-sufficiency signals, therefore, inhibit N uptake (Ruffel et al., 2011; Kiba and Krapp, 2016). No wonder, cytokinin mediated repression of NRT2.1 may also affect the lateral root development.

In a word, upregulation of NRT1.1 and NRT2.1 might be occurred in winter types due to local high nitrate concentration and mild nitrate deficiency which is a common feature of heterogeneous nitrate availability in the soil. Relative higher expression of these genes in winter type root system at 60d contributed to observed higher primary root branches in winter type comparing to spring type at 60d in the current study. Cytokinin signaling may repress or lower the NRT2.1 gene expression in spring type at 60d.

6.5.3.2. Abiotic stress related gene sets

Several gene sets related to different abiotic stresses were upregulated in both spring and winter types at both time points in GSEA. These gene sets were related to heat, cold, hypoxia, anoxia, drought, UV, reactive oxygen species etc. Interestingly, at 30d spring vs winter the number and type of abiotic stress related genes upregulated in winter and spring were somewhat similar. But the at 60d spring vs winter, the number of abiotic stress related gene sets upregulated in winter type was way higher than those of spring type. This higher number of upregulated abiotic stress related gene sets in 60d winter type may be due to pot blindness of the vigorous winter type root system. Several researcher previously suggested how plant growth and physiological activities were hampered due to pot size which is in other words due to pot blindness (Ismail et al., 1994; Bourgault et al., 2017; Dambreville et al., 2017; Sinclair et al., 2017). In the current research, we used large and long pots to eliminate the pot blindness. But the

root system of winter types at 60d were highly vigorous comparing to root system of winter types at 30d and spring types at 30d and 60d. Therefore, winter type root system may have experienced the pot blindness effect and, due to that subjected to stresses, had higher number of abiotic stress related gene sets up-regulated. Additionally, in GSEA and SNEA, several gene sets related to abiotic stress tolerance were up-regulated in winter types in 60d spring vs winter comparison. These gene sets were mostly related to drought tolerance, heat tolerance and detoxification process. This indicates that winter type root system may be able to withstand better when subjected to abiotic stresses by activating its defense response against those stresses.

6.5.3.3. Biotic stress related gene sets

Several other gene sets associated with plant defense response and wounding were differentially regulated in spring vs winter at both time points. Some of them were upregulated in spring type and some of them were upregulated in winter types. This suggests that both spring and winter types faced certain degree of microbe and/or insect pressure throughout the growth period and therefore the defense and wound related genes and gene sets were expressed in both types.

6.6. Conclusion

This research was conducted to assist in understanding of the gene regulatory networks underlying the root system variation between spring and winter types canola at later growth stages. Therefore, we compared the transcriptome changes between spring and winter types at two time points 30 days (no significant variation in root system of spring and winter) and 60 days (significant variation in root system of spring and winter). We detected cytokinin related genes and gene sets were differentially expressed exclusively at 60 days with an upregulation in

spring type. Based on this, we suggest cytokinin signaling might play a major role in inhibiting root growth in spring types at later growth stage. Although we did not detect any significant gibberellin related DEG in single gene analysis, gibberellin associated gene sets at 60 days were over-represented in spring types. We relate this fact to our observed root trait phenotypes in spring types showing elongated root length with lesser lateral root branch, a prime feature of gibberellin mediated root growth and development. Additional researches need to be conducted to confirm the role of cytokine and gibberellin in root growth and development in canola growth habits and their cross-talk with other phytohormones, especially auxin. The transcriptome profile developed in this study will serve as the basis of future research to elucidate physiological and biological processes involved in root growth variation in different canola/rapeseed growth habits.

CHAPTER 7. SUMMARY CONCLUSION AND FUTURE DIRECTION

In this research, we have followed two root phenotyping procedure, a modified version of mesocosm in the greenhouse and shovelomics in the field. Although shovelomics has been applied for large scale root phenotyping in few crops, mesocosm has only been applied for short scale root evaluation. We modified the mesocosm and applied it for large scale root phenotyping in the greenhouse which can be used in phenotyping roots in other crops. We compared different root architectural traits between spring and winter type canola at different time points after planting. We identified that there was no significant variation in root architectural traits at 30 days after planting. Variation starts between spring and winter type root system at 40 days after planting and it becomes highest at 60 days after planting. We also evaluated a set of spring and winter types canola under water stressed conditions and observed that the growth of winter type root system is more affected than the growth of spring type. We phenotyped the root architectural traits of 224 genotypes and performed a genome wide association study with ~37500 SNP markers under greenhouse conditions. All the root traits were found positively correlated with each other except the root angle (RA). Fifty-two significant marker loci associated with different root architectural traits were identified from this study. A total of 22 candidate genes for different root architectural traits within the neighboring regions of the markers were identified. Few of the candidate genes, *P-glycoprotein 6* (*PGP6*), *Tetraspanin 7* (*TET7*) and *ARABIDILLO-2* were located at the same physical position of the marker loci chrC03_12098594 (RL), chrA01_8813067 (PRB), and chrA04_rand_54410 (R_1Dia). In the field study, the root system architecture of216 genotypes were studied, and a genome wide association mapping with ~30,200 SNP markers was performed. We identified 31 significant marker loci

associated with different root traits and 15 candidate genes for root traits that were closely located with the significant markers. We combined all the significant marker loci from the greenhouse and field and assigned them on chromosomes of *B. napus* on the basis of physical locations of the markers. We have detected some regions on chromosome A06, A09, C01, C03, C05 and C06 where multiple markers from field and greenhouse associated with different root architectural traits were co-localized (Fig 7.1).

In phenotypic analysis, root diameters are positively correlated with other root traits, and several significant marker loci were co-localized in the same physical region controlling soil level root diameter, primary root branches and root vigor score. Therefore, we suggest that taproot root may play an important role in overall root growth and it can be a proxy trait for other root architectural traits. The candidate genes identified in this research can be a good target for mutant study through reverse genetics approach. Additionally, we compared the transcriptomes identified in spring and winter root systems at 30 days and 60 days and detected significantly differentially expressed genes between these two growth habits that might cause the root system variation between them. Several cytokinin and gibberellin related genes and genes sets were significantly differentially expressed in spring type at 60 days. Cytokinin inhibits the root growth and development in many crops and gibberellin plays an important role in reducing lateral root growth. Based on these, we suggest that cytokinin and gibberellin may have some important roles in inhibiting root growth in spring type at later growth stages which leads to the root system variation between spring and winter types. Extensive gene expression data generated in this research will further assist to understand the natural variation of root system in canola growth habits.

In addition, we have compared the gene models within 50 Kbp and 100Kbp upstream and downstream of the significant marker loci from greenhouse and field condition, respectively with the gene models of significantly differentially expressed transcripts from our transcriptome study. We have identified, total twelve common gene models that were detected in both GWAS and transcriptome study (Table 7.1). Four of the gene models were encoding three root related candidate genes GASA4, NRT1.1 and NRT2.1.

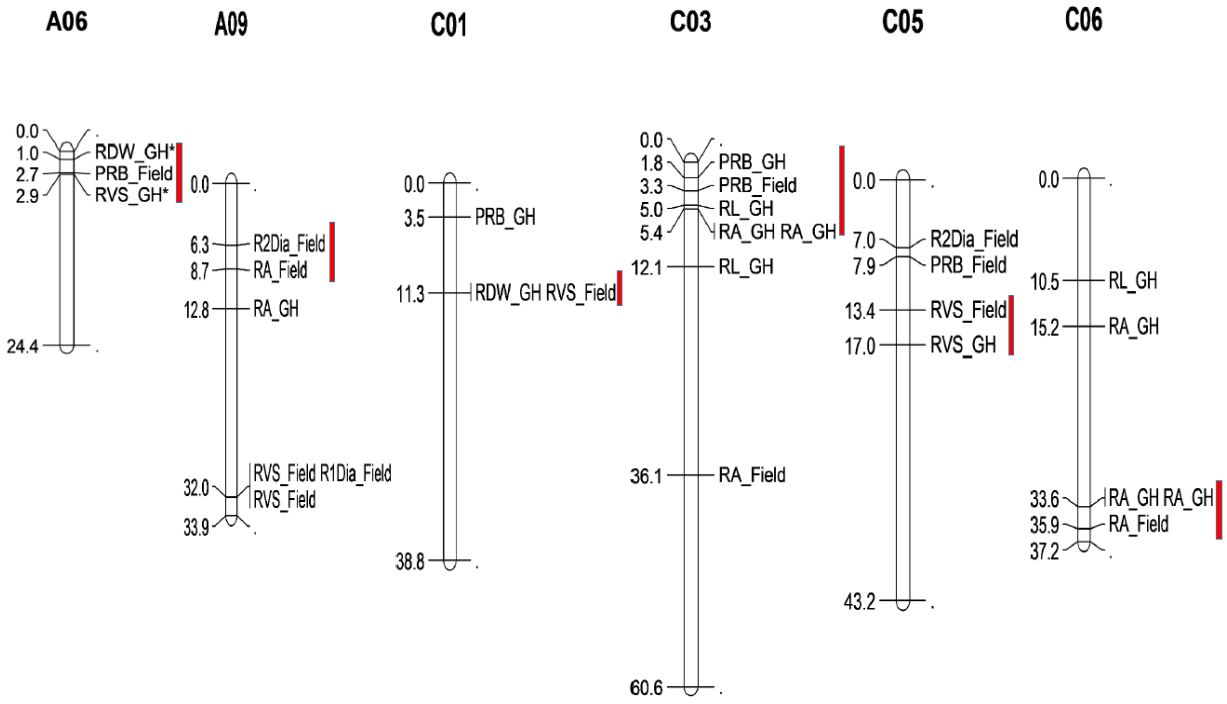


Figure 7.1. Significant markers from field and greenhouse neighboring on the same chromosome

Table 7.1. Common candidate genes after combining all the gene models from GWAS and transcriptome study.

Marker	Traits	Datasets	BNA gene model	AT gene model	Name	Significantly Differentially expressed at	Expression direction at 30d & 60d
chrC03_3328679	**PRB**	**2015_F**	**BnaC03g06850**	**AT5G15230**	**GAST1 protein homolog 4**	**30 Days**	**Down Down**
chrC09_19217839	RS	2015_F	BnaC09g21960	AT2G03550	alpha/beta-Hydrolases superfamily protein	30 Days	UpUp
chrA02_1108743	**PRB**	**2015_GH**	**BnaA02g02560**	**AT5G15230**	**GAST1 protein homolog 4**	**60 Days**	**UpUp**
chrA07_22509933	R1Dia	2016_GH	BnaA07g32700	AT1G76410	RING/U-box superfamily protein	60 Days	DownUp
chrC01_11247236	RDW	2015_GH, Comb_GH	BnaC01g16280	AT4G24340	Phosphorylase superfamily protein	60 Days	UpUp
chrC04_2352267	PRB	2016_GH	BnaC04g03300	AT2G44065	Ribosomal protein L2 family	60 Days	DownDown
chrA02_22847077	RA	2015_F, Comb_F	BnaA02g31810	AT5G25940	early nodulin-related	60 Days	UpDown
chrA06_2653673	**PRB**	**Comb._F**	**BnaA06g04560**	**AT1G08090**	**nitrate transporter 2:1**	**60 Days**	**DownDown**
chrA09_32034416	**R1Dia, RS**	**2016_F(R1Dia), Comb._F(RS)**	**BnaA09g47380**	**AT1G12110**	**nitrate transporter 1.1**	**60 Days**	**DownDown**
BnaC06g38360D	RA	Comb._F	BnaC06g38140	AT1G77480	Eukaryotic aspartyl protease family protein	60 Days	DownDown
chrC03_1822934	PRB	2015_GH, Comb._GH	BnaC03g03830	AT5G09500	Ribosomal protein S19 family protein	Both	DownDown
chrA02_22847077	RA	2015_F, Comb._F	BnaA02g31600	AT5G26280	TRAF-like family protein	Both	UpUp

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APPENDIX

Table A.1. List of accessions used in this study.

Accession name	Country of origin/obtained	Growth Habit
AR91004	USA	Winter
ARC 90016[ab]	USA	Winter
ARC 97018	USA	Winter
ARC 97019[ab]	USA	Winter
ARC-2180-1[b]	USA	Winter
Aspen[b]	USA	Winter
Aviso	Canada	Winter
Azuma	South Korea	Semi-winter
Azumasho[ab]	South Korea	Semi-winter
Baraska	Germany	Winter
Barkant	Netherlands	Winter
Barplina	South Korea	Winter
Beryl	Poland	Winter
Billy	Sweden	Winter
Bingo	USA	Spring
BNW 161/83	Germany	Winter
BO-63	Canada	Spring
Bolko[ab]	Poland	Winter
Bridger	USA	Winter
Brink[ab]	Sweden	Winter
Brio	France	Spring
Bronowski[ab]	Poland	Spring
Buk Wuk 3	South Korea	Spring
Cascade	USA	Winter
Celebra	Sweeden	Spring
Ceskia Tabor	Czechoslovakia	Spring
CHUN-NUNG 1	China	Winter
Cobra	Germany	Winter
Colt	USA	Spring
Colza	South Korea	Spring
Colza 18 Miroc	South Korea	Semi-winter
Comet[b]	Sweden	Spring
Conquest	Canada	Spring
Corvette	UK	Winter

Table A.1. List of accessions used in this study (Continued)

Accession name	Country of origin/obtained	Growth Habit
Cougar	Canada	Spring
CR 742/91	Germany	Rutabaga
Crop	France	Spring
Crystal	Sweden	Winter
Cult	Canada	Winter
Czyzowski[ab]	Poland	Spring
Da vinci[b]	Canada	Winter
Dae cho sen	South Korea	Semi-winter
Darmar	France	Winter
Delta	Sweden	Spring
Doon Major[ab]	USA	Rutabaga
Doon Major Swede	New Zealand	Winter
Drakkar	France	Spring
Drawft[ab]	South Korea	Winter
Eckendorfer Mali	South Korea	Semi-winter
Eragi	Germany	Winter
Ericka	USA	Winter
Erra	Germany	Winter
Evvin	Russian Federation	Spring
Expander	Germany	Winter
Fashion	Canada	Winter
Fertodi	South Korea	Winter
Fonto	South Korea	Spring
France 1	France	Spring
Fuji	South Korea	Spring
Galant	Serbia	Spring
Galaxy[b]	Sweden	Spring
Galileo	Canada	Winter
Gebr Dippes	South Korea	Winter
Gido	Germany	Spring
Girita	Germany	Semi-winter
Gisora	Germany	Spring
Glacier	Sweden	Winter
Global	Sweden Malmohus	Spring
Golden	Canada	Spring
Gora	Germany	Spring
Goya[b]	Canada	Winter
Gulle	Sweden	Spring

Table A.1. List of accessions used in this study (Continued)

Accession name	Country of origin/obtained	Growth Habit
Gullivar	Sweden	Spring
Hamburg	South Korea	Winter
Helga	Germany	Semi-winter
Hi-Q	Canada	Spring
Host rape regel	South Korea	Winter
Ibiza	Canada	Winter
IR-2	Hungary	Spring
Iwashiro-natane[ab]	South Korea	Winter
Janetzkis	South Korea	Spring
Jasna	Serbia	Spring
Jupiter	USA	Winter
Kanada	Poland	Spring
Karafuto	South Korea	Winter
Kasuya	South Korea	Winter
Kasuyashu	South Korea	Winter
Klinki	South Korea	Spring
Korina	Germany	Winter
Kosa	Germany	Spring
Koubunab	South Korea	Spring
Kovalevskjj	Ukraine	Spring
Kraphhauser	South Korea	Spring
Kritmar rape[ab]	South Korea	Spring
KS3579	USA	Winter
KSU 1		
KSU 10	USA	Winter
KSU 2	USA	Winter
KSU 3	USA	Winter
KSU 4	USA	Winter
KSU 5	USA	Winter
KSU 7	USA	Winter
KSU 8	USA	Winter
Kuju	South Korea	Winter
Kutkowski	South Korea	Winter
Ladoga	Canada	Winter
Laura	Germany	Spring
Legend	Sweden	Spring
Lembkes	South Korea	Winter
Lembkes malchower	South Korea	Winter
Lenora	South Korea	Winter

Table A.1. List of accessions used in this study (Continued)

Accession name	Country of origin/obtained	Growth Habit
Lesira	Germany	Winter
Lester	Germany	Winter
Librador	Germany	Winter
Licantara	Germany	Winter
Lieikoposki	South Korea	Semi-winter
Lifura	South Korea	Spring
Lindora-00	Germany	Winter
Lindore	Germany	Winter
Linglandor	Germany	Winter
Linus	South Korea	Winter
Lirabou	Germany	Winter
Liradonne	Germany	Winter
Liratrop	Germany	Winter
Lisora	Germany	Semi-winter
Lorenze	Canada	Winter
Major	France	Semi-winter
Marcus[b]	South Korea	Winter
Mar'janovskija[b]	Ukraine	Spring
Midas	Canada	Spring
Miekuro Dane	South Korea	Spring
Mihonatane	South Korea	Winter
Miochowski[b]	France	Semi-winter
Mlochowski	Poland	Semi-winter
Mulchower	South Korea	Winter
Murame nadame	South Korea	Semi-winter
Mutsumi[ab]	Japan	Semi-winter
N001-28-246-5-4	South Korea	Semi-winter
NDSU0472	USA	Spring
NDSU0473[ab]	USA	Spring
NDSU0474[ab]	USA	Spring
NDSU0619	USA	Spring
NDSU0620	USA	Spring
NDSU0728	USA	Spring
NDSU0729	USA	Spring
NDSU10999	USA	Spring
NDSU151000	Canada	Spring
NDSU15989	USA	Spring
NDSU161013	USA	Spring

Table A.1. List of accessions used in this study (Continued)

Accession name	Country of origin/obtained	Growth Habit
NDSU31011	USA	Spring
NDSU41000	USA	Spring
NDSU7997	USA	Spring
NDSU81000[b]	USA	Spring
NDSU91013	USA	Spring
Niedera-rubacher[b]	South Korea	Winter
Nilla 1022	South Korea	Semi-winter
Nilla glossy[ab]	South Korea	Semi-winter
Norin#1	Japan	Winter
NU 41737	Turkey	Spring
NU 51084	Sweden	Spring
Nugget	South Korea	Semi-winter
NY-10[ab]	China	Semi-winter
NY-18	China	Winter
NY-20	China	Semi-winter
NY-7	China	Semi-winter
NY-8	China	Semi-winter
Oleifera	South Korea	Semi-winter
Oro	Canada	Spring
Orpal	France	Spring
Peace	Canada	Spring
Petanova-lihonova	South Korea	Semi-winter
Polo canola	USA	Spring
Premier[b]	USA	Spring
Printol	USA	Spring
Prota[ab]	Germany	Spring
Prover	USA	Winter
Q2[ab]	Canada	Spring
R. Creaus	South Korea	Winter
Rafal	France	Winter
Ramses[b]	South Korea	Winter
Rang[b]	South Korea	Semi-winter
Ratnik[ab]	Serbia	Spring
Rebel	USA	Semi-winter
Red Russian[b]	USA	Winter
Regal	South Korea	Winter
Regent	Canada	Spring

Table A.1. List of accessions used in this study (Continued)

Accession name	Country of origin/obtained	Growth Habit
Regina II	Canada	Spring
Reston	USA	Spring
Rico	Germany	Spring
Ridana[b]	Germany	Winter
Romeo	France	Spring
Russia 5	Russian Federation	Spring
Seoul	South Korea	Spring
Sera[b]	Germany	Semi-winter
Siberian[ab]	USA	Winter
Silex	Canada	Spring
Sparta	USA	Winter
Sunrise	USA	Spring
Sval of Gullen	South Korea	Spring
Synra	South Korea	Winter
Taichang	South Korea	Semi-winter
Tanka	South Korea	Semi-winter
Tanto Fu 85	France	Spring
Titus	South Korea	Winter
Tokiwa	South Korea	Semi-winter
Tonus[b]	South Korea	Spring
Topasb	Sweden	Spring
Tower	Canada	Spring
Trebicska[b]	Czech Republic	Winter
Tri-Bridger[b]	USA	Winter
Turret[ab]	Canada	Spring
Ujfertodi[ab]	Hungary	Winter
Vanda[ab]	Germany	Winter
Visiona	Canada	Winter
Vostochno-sibirskii	Russian Federation	Spring
Wasefuji[ab]	South Korea	Spring
Weal dong cho	South Korea	Semi-winter
Westar	Canada	Spring
Wichita[b]	USA	Winter
Willa	South Korea	Spring
Wipol	Norway	Semi-winter
Wira	Germany	Winter
Yong dang	South Korea	Semi-winter

Table A.1. List of accessions used in this study (Continued)

Accession name	Country of origin/obtained	Growth Habit
Yonkkaichi kwo[b]	South Korea	Semi-winter

[a]Accessions not included for RL, PRB, RA and RVS

[b]Accessions not included for RDW

Table A.2. Validation of the significant markers (0.01) for primary root branches (PRB) by genotype based prediction of the germplasms.

Germplasms	PRB											
	chrC04_2352222	chrC04_2352228	chrC04_2352267	PRED	OBS	chrA01_8813067	PRED	OBS	chrC03_1822934	PRED	OBS
	C/T	A/G	T/A			G/T			G/T		
Drawft	C	A	T	H	MH	G	H	MH	K	M	MH
Bronowski	C	A	T	H	ML*	G	H	ML*	G	H	ML*
Iwawoochi	C	A	T	H	MH	G	H	MH	G	H	MH
Q2	Y	R	W	M	MH	G	H	MH	G	H	MH
Ratnik	Y	R	W	M	H*	G	H	H	G	H	H
Attila	C	A	T	H	ML*	G	H	ML*	T	L	ML
Bolko	C	A	T	H	MH	G	H	MH	G	H	MH
Nemercanskjj_2268	C	A	T	H	MH	K	M	MH	G	H	MH
Siberian	C	A	T	H	H	G	H	H	G	H	H
Yonkkaichi_kwo	Y	R	W	M	ML	G	H	ML*	T	L	ML
NDSU0474	C	A	T	H	MH	G	H	MH	T	L	MH*
ARC_90016	C	A	T	H	H	G	H	H	T	L	H*
Brink	C	A	T	H	MH	G	H	MH	G	H	MH
Czyzowskich	C	A	T	H	H	G	H	H	G	H	H
Mar_160059	C	A	T	H	MH	G	H	MH	G	H	MH
Tonus	C	A	T	H	H	G	H	H	G	H	H
Vanda	C	A	T	H	H	G	H	H	G	H	H
Wasefuji	T	A	A	H	MH	K	M	MH	G	H	MH
Prota	C	A	T	H	H	G	H	H	K	M	H
Ramses	C	A	T	H	MH	G	H	MH	G	H	MH

**Did not matched with our prediction, PRED=Predicted phenotype, OBS=Observed phenotype, H=High, MH= Moderately high, ML= Moderately low, L=Low, Y,R,WK=heterozugous for the markers.*

Table A.3. Validation of the significant markers (0.01) for root vigor score (RVS), and root length (RL) by genotype based prediction of the germplasms.

Germplasms	RVS						RL		
	chrA04_5977698	PRED	OBS	chrA01_7949816	PRED	OBS	chrC03_4976549	PRED	OBS
	T/C			A/G			A/G		
Drawft	T	H	MH	G	L	MH*	A	H	MH
Bronowski	T	H	MH	R	M	MH	A	H	MH
Iwawoochi	T	H	MH	A	H	MH	A	H	MH
Q2	T	H	ML*	R	M	ML*	A	H	MH
Ratnik	T	H	MH	A	H	MH	A	H	H
Attila	T	H	MH	A	H	MH	A	H	MH
Bolko	T	H	ML*	A	H	ML*	A	H	MH
Nemercanskjj_2268	T	H	MH	R	M	MH	A	H	MH
Siberian	T	H	MH	A	H	MH	A	H	H
Yonkkaichi_kwo	T	H	MH	A	H	MH	A	H	MH
NDSU0474	T	H	ML*	A	H	ML*	A	H	H
ARC_90016	T	H	ML*	A	H	ML*	A	H	H
Brink	T	H	MH	R	M	MH	A	H	MH
Czyzowskich	T	H	H	A	H	H	A	H	H
Mar_160059	T	H	MH	R	M	MH	A	H	ML*
Tonus	T	H	MH	A	H	MH	G	L	MH*
Vanda	T	H	H	A	H	H	A	H	MH
Wasefuji	T	H	MH	A	H	MH	A	H	H
Prota	T	H	MH	A	H	MH	A	H	H
Ramses	Y	M	H*	A	H	H	A	H	H

**Did not matched with our prediction, PRED=Predicted phenotype, OBS=Observed phenotype, H=High, MH= Moderately high, ML= Moderately low, L=Low, Y,R,WK=heterozugous for the markers.*

Table A.4. Validation of the significant markers (0.01) for root angle (RA) by genotype-based prediction of the germplasms.

Germplasms	RA							
	chrC06_33610701	chrC06_33610722	PRED	OBS	chrC03_5436381	chrC03_5436391	PRED	OBS
	T/G	T/G			T/G	C/T		
Drawft	T	T	H	MH	T	C	H	MH
Bronowski	T	T	H	H	T	C	H	H
Iwawoochi	T	T	H	MH	T	C	H	MH
Q2	T	T	H	MH	T	C	H	MH
Ratnik	T	T	H	H	T	C	H	H
Attila	T	T	H	MH	T	C	H	MH
Bolko	T	T	H	MH	T	C	H	MH
Nemercanskjj_2268	T	T	H	ML*	T	C	H	ML*
Siberian	T	T	H	MH	K	Y	M	MH
Yonkkaichi_kwo	T	T	H	H	T	C	H	H
NDSU0474	T	T	H	H	T	C	H	H
ARC_90016	T	T	H	H	T	C	H	H
Brink	T	T	H	MH	T	C	H	MH
Czyzowskich	K	T	H/M	MH	T	C	H	MH
Mar_160059	K	K	M	ML	K	Y	M	ML*
Tonus	K	K	M	MH	K	Y	M	MH
Vanda	T	T	H	MH	K	Y	M	MH
Wasefuji	G	G	L	MH*	T	C	H	MH
Prota	T	T	H	MH	T	C	H	MH
Ramses	T	T	H	MH	T	C	H	MH

**Did not matched with our prediction, PRED=Predicted phenotype, OBS=Observed phenotype, H=High, MH= Moderately high, ML= Moderately low, L=Low, Y,R,WK=heterozugous for the markers.*

Table A.5. Name, origin and growth habits of the 216 germplasm accessions used in this study

Name of the Accessions	PI number	Country of origin/obtained	Growth Habit
Doon Major	PI 649143	USA	Rutabaga
Bristol White	NSL 22937	USA	Rutabaga
Gylle	PI 469812	South Korea	Semi-winter
Major	PI 469891	France	Semi-winter
NY-20	#N/A	China	Semi-winter
NY-8	#N/A	China	Semi-winter
Sera	PI 458957	Germany	Semi-winter
Tokiwa	PI 470049	South Korea	Semi-winter
Wipol	PI 535871	Norway	Semi-winter
Azuma	PI 469730	South Korea	Semi-winter
Dae cho sen	PI 469758	South Korea	Semi-winter
Girita	PI 458947	Germany	Semi-winter
Helga	PI 649136	Germany	Semi-winter
Lisora	PI 458953	Germany	Semi-winter
Matador	PI 469899	South Korea	Semi-winter
Mlochowski	PI 535848	Poland	Semi-winter
Murame nadame	PI 469940	South Korea	Semi-winter
NY-7	#N/A	China	Semi-winter
Nabo	PI 469944	South Korea	Semi-winter
Nilla glossy	PI 469946	South Korea	Semi-winter
Todane	PI 470048	South Korea	Semi-winter
Yong dang	PI 470060	South Korea	Semi-winter
Yonkkaichi kwo	PI 470061	South Korea	Semi-winter
Shang you	PI 391553	China	Semi-winter
Azumasho	PI 469734	South Korea	Semi-winter
Colza 18 Miroc	PI 469757	South Korea	Semi-winter
Eckendorfer Mali	PI 469784	South Korea	Semi-winter
Lieikoposki	PI 469887	South Korea	Semi-winter
Miochowski	PI 469902	France	Semi-winter
Mutsumi	PI 469942	Japan	Semi-winter
N001-28-246-5-4	PI 469943	South Korea	Semi-winter
Nilla 1022	PI 469947	South Korea	Semi-winter
Petanova-lihonova	PI 470003	South Korea	Semi-winter
Su weon chag	PI 470031	South Korea	Semi-winter
Taichang	PI 470036	South Korea	Semi-winter
Tanka	PI 470044	South Korea	Semi-winter
Nugget	PI 469999	South Korea	Semi-winter

Table A.5. Name, origin and growth habits of the 216 germplasm accessions used in this study (Continued)

Name of the Accessions	PI number	Country of origin/obtained	Growth Habit
Rang	PI 470013	South Korea	Semi-winter
Rebel	PI 540457	USA	Semi-winter
Oleifera	PI 470000	South Korea	Semi-winter
NY-10	#N/A	China	Semi-winter
Bingo	PI 546468	USA	Spring
Colza	PI 469756	South Korea	Spring
Comet	Ames 15939	Sweden	Spring
Conquest	#N/A	Canada	Spring
Cougar	#N/A	Canada	Spring
Drakkar	#N/A	France	Spring
Galant	#N/A	Serbia	Spring
Gido	PI 458946	Germany	Spring
Bronowski	PI 469737, Ames 22548, PI 649132	Poland	Spring
Golden	PI 649126	Canada	Spring
Gullivar	PI 458937	Sweden	Spring
Hi-Q	#N/A	Canada	Spring
Kanada	#N/A	Poland	Spring
Kovalevskjj	PI 633132	Ukraine	Spring
Legend	PI 633118	Sweden	Spring
Lifura	PI 469888	South Korea	Spring
Ratnik	#N/A	Serbia	Spring
Romeo	PI 458971	France	Spring
Silex	#N/A	Canada	Spring
Topas	PI 601201	Sweden	Spring
Tower	PI 431574, Ames 2792, PI 431574	Canada	Spring
Vostochno-sibirskii	PI 633126	Russian Federation	Spring
Westar	Ames 26653	Canada	Spring
Willa	PI 470058	South Korea	Spring
NDSU151000	#N/A	Canada	Spring
Delta	PI 543937	Sweden	Spring
Evvin	PI 633131	Russian Federation	Spring
Fonto	PI 469789	South Korea	Spring
France 1	PI 469791	France	Spring
Galaxy	Ames 15938	Sweden	Spring

Table A.5. Name, origin and growth habits of the 216 germplasm accessions used in this study (Continued)

Name of the Accessions	PI number	Country of origin/obtained	Growth Habit
Gora	PI 458949	Germany	Spring
Janetzkis	PI 469826	South Korea	Spring
Klinki	PI 469840	South Korea	Spring
Brio	PI 458919	France	Spring
Kosa	PI 458951	Germany	Spring
Koubun	PI 469841	South Korea	Spring
Kritmar rape	PI 469843	South Korea	Spring
Kraphhauser	PI 469842	South Korea	Spring
Midas	PI 431571	Canada	Spring
Miekuro Dane	PI 469901	South Korea	Spring
Oro	PI 458930	Canada	Spring
Orpal	PI 458968	France	Spring
Polo canola	Ames 26635	USA	Spring
Regent	PI 431572	Canada	Spring
Seoul	PI 537090	South Korea	Spring
Ceskia Tabor	Ames 2793	Czechoslovakia	Spring
Sunrise	PI 597352	USA	Spring
NDSU0472	#N/A	USA	Spring
NDSU0473	#N/A	USA	Spring
NDSU0474	#N/A	USA	Spring
NDSU0619	#N/A	USA	Spring
NDSU0620	#N/A	USA	Spring
NDSU0728	#N/A	USA	Spring
NDSU0729	#N/A	USA	Spring
NDSU10999	#N/A	USA	Spring
NDSU15989	#N/A	USA	Spring
NDSU161013	#N/A	USA	Spring
NDSU31011	#N/A	USA	Spring
NDSU41000	#N/A	USA	Spring
NDSU7997	#N/A	USA	Spring
NDSU91013	#N/A	USA	Spring
BO-63	Ames 15651	Canada	Spring
Cresor	PI 458920	France	Spring
Crop	PI 458922	France	Spring
Czyzowski	PI 535847, PI 311728	Poland	Spring
Tanto Fu 85	#N/A	France	Spring

Table A.5. Name, origin and growth habits of the 216 germplasm accessions used in this study (Continued)

Name of the Accessions	PI number	Country of origin/obtained	Growth Habit
Fuji	PI 469801	South Korea	Spring
IR-2	PI 531280	Hungary	Spring
Regina II	Ames 1669	Canada	Spring
Sval of Gullen	PI 470033	South Korea	Spring
NU 51084	PI 633124	Sweden	Spring
Tonus	PI 470050	South Korea	Spring
Turret	PI 365644	Canada	Spring
Wasefuji	PI 470054	South Korea	Spring
Celebra	PI 538766	Sweeden	Spring
Mar'janovskij	PI 633125	Ukraine	Spring
NU 41737	PI 649135	Turkey	Spring
Printol	PI 552810	USA	Spring
Reston	PI 649152	USA	Spring
Premier	PI 639274	USA	Spring
Prota	PI 458955	Germany	Spring
Rico	PI 458956	Germany	Spring
Russia 5	PI 470021	Russian Federation	Spring
Global	PI 601200	Sweden Malmohus	Spring
Jasna	#N/A	Serbia	Spring
Baraska	PI 649137	Germany	Winter
Barkant	PI 531274	Netherlands	Winter
Crystal	PI 601261	Sweden	Winter
Glacier	PI 601260	Sweden	Winter
Iwawoochi	PI 469823	South Korea	Winter
Jupiter	Ames 6100	USA	Winter
Karafuto	PI 469829	South Korea	Winter
Korina	PI 535856	Germany	Winter
KSU 6	#N/A	USA	Winter
Lembkes malchower	PI 469885	South Korea	Winter
Lesira	PI 409023	Germany	Winter
Lester	PI 535857	Germany	Winter
Lindora-00	PI 601282	Germany	Winter
Linglandor	PI 531283	Germany	Winter
Lirama	PI 535860	Germany	Winter
Liratrop	PI 531284	Germany	Winter
Regal	PI 470019	South Korea	Winter

Table A.5. Name, origin and growth habits of the 216 germplasm accessions used in this study (Continued)

Name of the Accessions	PI number	Country of origin/obtained	Growth Habit
Titus	PI 470046	South Korea	Winter
Trebicska	PI 399418, PI 470052	Czech Republic	Winter
Tri-Bridger	PI 542984	USA	Winter
Valdor	PI 535850	France	Winter
Wichita	PI 612846	USA	Winter
Beryl	PI 535851	Poland	Winter
Cobra	PI 601661	Germany	Winter
Cult	#N/A	Canada	Winter
Da vinci	#N/A	Canada	Winter
BNW 161/83	PI 531275	Germany	Winter
Dong Buk	PI 469759	South Korea	Winter
Erra	PI 409022	Germany	Winter
Expander	PI 469787	Germany	Winter
Fashion	#N/A	Canada	Winter
Fertodi	PI 469788	South Korea	Winter
Bolko	PI 633120	Poland	Winter
Gebr Dippes	PI 469802	South Korea	Winter
Bridger	PI 509073	USA	Winter
Ibiza	#N/A	Canada	Winter
KSU 1	#N/A	USA	Winter
KSU 2	#N/A	USA	Winter
KSU 5	#N/A	USA	Winter
Kasuyashu	PI 469831	South Korea	Winter
Kuju	PI 469845	South Korea	Winter
Kutkowski	PI 469882	South Korea	Winter
Ladoga	#N/A	Canada	Winter
Lenora	PI 469886	South Korea	Winter
Librador	PI 531281	Germany	Winter
Linus	PI 469889	South Korea	Winter
Lorenze	#N/A	Canada	Winter
NY-18	#N/A	China	Winter
Siberian	Ames 26626	USA	Winter
Sparta	PI 649141	USA	Winter
Synra	PI 470035	South Korea	Winter
Wira	PI 458959	Germany	Winter
ARC-2180-1	#N/A	USA	Winter

Table A.5. Name, origin and growth habits of the 216 germplasm accessions used in this study (Continued)

Name of the Accessions	PI number	Country of origin/obtained	Growth Habit
ARC 97019	#N/A	USA	Winter
Bienvenu	Ames 15654	USA	Winter
AR91004	PI 610258	USA	Winter
ARC 90016	#N/A	USA	Winter
Barplina	PI 469736	South Korea	Winter
Brink	PI 458935	Sweden	Winter
Eragi	PI 458945	Germany	Winter
Host rape regel	PI 469817	South Korea	Winter
Kasuya	PI 469830	South Korea	Winter
KS3579	PI 594321	USA	Winter
KSU 7	#N/A	USA	Winter
KSU 8	#N/A	USA	Winter
KSU 9	#N/A	USA	Winter
Lembkes	PI 469883	South Korea	Winter
Licantara	PI 535874	Germany	Winter
Lindore	PI 531282	Germany	Winter
Lirabou	PI 535858	Germany	Winter
Mihonatane	PI 469903	South Korea	Winter
Mulchower	PI 469892	South Korea	Winter
Niedera-rubacher	PI 469945	South Korea	Winter
Norin#1	PI 469949	Japan	Winter
CHUN-NUNG 1	PI 391552	China	Winter
Corvette	PI 555467	UK	Winter
Per	PI 649128, Ames 26657	Sweden	Winter
Prover	PI 649144	USA	Winter
Ridana	PI 535865	Germany	Winter
R. Creaus	PI 470010	South Korea	Winter
Ramses	PI 470012	South Korea	Winter
Red Russian	Ames 26645	USA	Winter
Rafal	PI 458970	France	Winter
KSU 3	#N/A	USA	Winter
KSU 10	#N/A	USA	Winter
Billy	#N/A	Sweden	Winter
Vision	#N/A	Canada	Winter
KSU 4	#N/A	USA	Winter
ARC 97018	#N/A	USA	Winter

Table A.5. Name, origin and growth habits of the 216 germplasm accessions used in this study (Continued)

Name of the Accessions	PI number	Country of origin/obtained	Growth Habit
Goya	#N/A	Canada	Winter

Table A.6. Differentially regulated gene sets in spring vs winter at 30 days detected from Gene Set Enrichment Analysis (GSEA)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in winter	No. of measured entities	p-value
response to high light intensity	27	0.00017	structural constituent of ribosome	186	1.5E-10
ADP binding	21	0.00025	translation	187	1E-08
extracellular region	373	0.00025	ribosome	149	8E-08
defense response	151	0.00045	plasmodesma	355	2.1E-06
response to jasmonic acid	60	0.00064	cytosolic ribosome	114	2.3E-06
amino acid transmembrane transporter activity	18	0.00084	cell wall	195	2.8E-06
peptidase S10 family	8	0.00089	cytosolic small ribosomal subunit	47	5.5E-06
serine carboxypeptidase	8	0.00089	cytosolic large ribosomal subunit	64	3.6E-05
serine-type carboxypeptidase activity	8	0.00089	extracellular region	358	0.00016
response to wounding	83	0.00122	nucleolus	164	0.00021
polyamine biosynthetic process	5	0.0017	lactoperoxidase	25	0.00039
ribosome	62	0.0019	lactoperoxidase	25	0.00039
cell wall	168	0.00271	lactoperoxidase	25	0.00039
calcium ion transmembrane transport	10	0.00273	lactoperoxidase	25	0.00039
cytosolic small ribosomal subunit	30	0.00306	ribosome biogenesis	77	0.0004
SNAP receptor activity	19	0.00373	mitochondrial proton-transporting ATP synthase complex, catalytic core F(1)	6	0.00047
monooxygenase activity	19	0.00417	hydrogen peroxide catabolic process	32	0.00079
Plant Growth Auxin Signaling	15	0.0043	translational elongation	31	0.00079
amino acid transport	15	0.005	Apoplast	166	0.00088
phenylpropanoid biosynthetic process	7	0.00539	hydrogen-transporting ATP synthase, F1 sector	5	0.00107

Table A.6. Differentially regulated gene sets in spring vs winter at 30 days detected from Gene Set Enrichment Analysis (GSEA) (Continued)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in winter	No. of measured entities	p-value
chitin catabolic process	7	0.00649	ribosomal protein L12P family	5	0.00111
chitin binding	7	0.0066	ribosomal protein L12P family	5	0.00111
response to heat	78	0.00726	mitochondrial proton-transporting ATP synthase complex	11	0.00139
cysteine biosynthetic process from serine	6	0.00868	defense response to virus	8	0.00172
SNARE binding	17	0.0088	heme binding	75	0.0019
response to other organism	18	0.00916	copper ion binding	106	0.00229
chloroplast membrane	25	0.00967	ubiquitin-like domain	16	0.00271
chitinase activity	6	0.01004	peroxidase activity	43	0.0028
cellular response to heat	8	0.01029	endopeptidase inhibitor activity	5	0.00286
vesicle fusion	15	0.0104	small heat shock protein	7	0.00419
positive regulation of flower development	10	0.01137	small heat shock protein	7	0.00419
cysteine biosynthetic process	9	0.01147	response to wounding	66	0.00451
small heat shock protein	11	0.01164	ADP binding	13	0.0049
small heat shock protein	11	0.01164	endoplasmic reticulum lumen	22	0.00494
SNARE complex	18	0.01177	large ribosomal subunit	28	0.00506
cysteine-type endopeptidase inhibitor activity	5	0.01183	response to oxidative stress	124	0.00535
cytosolic ribosome	68	0.01211	monooxygenase activity	10	0.00542
AMP salvage	5	0.01216	response to brassinosteroid	7	0.00585
UDP-glycosyltransferase activity	18	0.01282	Vacuole	259	0.00594

Table A.6. Differentially regulated gene sets in spring vs winter at 30 days detected from Gene Set Enrichment Analysis (GSEA) (Continued)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in winter	No. of measured entities	p-value
response to bacterium	38	0.01345	chitinase activity	7	0.006
cellular response to nitrogen starvation	7	0.01382	unsaturated fatty acid biosynthetic process	5	0.00743
translation	93	0.01548	defense response to fungus	64	0.00749
response to salicylic acid	48	0.01651	chitin catabolic process	6	0.00755
symporter activity	19	0.01655	defense response	121	0.00801
hydrolase activity, hydrolyzing O-glycosyl compounds	34	0.01673	proton-transporting ATP synthase activity, rotational mechanism	11	0.00866
metabolic process	47	0.01675	water channel	8	0.0095
sucrose biosynthetic process	5	0.01681	water channel	8	0.0095
plasmodesma	360	0.01715	response to molecule of bacterial origin	7	0.00995
structural constituent of ribosome	93	0.01742	L-phenylalanine catabolic process	5	0.01
protein autophosphorylation	47	0.01759	LIM domain	6	0.01025
Ca2+-transporting ATPase	7	0.01766	response to heat	64	0.01026
root hair cell development	5	0.01774	polysaccharide catabolic process	5	0.01047
gravitropism	7	0.01806	Jasmonic Acid, Ethylene, and Salicylic Acid Crosstalk Signaling	18	0.01055
IAAs	11	0.01824	acid phosphatase activity	6	0.01084
Osmotic Stress Signaling	6	0.01861	integral component of plasma membrane	44	0.01089
Gravitropism and Phototropism Auxin Signaling	11	0.0195	water transport	7	0.01155

Table A.6. Differentially regulated gene sets in spring vs winter at 30 days detected from Gene Set Enrichment Analysis (GSEA) (Continued)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in winter	No. of measured entities	p-value
cellulose synthase (UDP-forming)	9	0.01965	microtubule binding	10	0.0119
cellulose synthase (UDP-forming)	9	0.01965	viral process	17	0.01215
cellulose synthase (UDP-forming)	9	0.01965	calcium-transporting ATPase activity	5	0.01217
cellulose synthase activity	9	0.01965	Fe2OG dioxygenase domain	10	0.01226
calcium-transporting ATPase activity	8	0.01967	glucosinolate catabolic process	8	0.01252
proteolysis involved in cellular protein catabolic process	24	0.02091	fatty acid desaturase family	7	0.01304
killing of cells of other organism	6	0.02253	CLE peptide family	5	0.01314
response to stress	27	0.02298	response to auxin	67	0.01482
negative regulation of translation	5	0.02352	cell growth	9	0.01612
cobalt ion binding	28	0.0239	response to jasmonic acid	45	0.01617
glucan endo-1,3-beta-D-glucosidase	5	0.02403	water channel activity	13	0.0179
glucan endo-1,3-beta-D-glucosidase activity	5	0.02403	oxidation-reduction process	359	0.01883
nutrient reservoir activity	12	0.02432	cation transport	7	0.01901
calcium channel activity	5	0.02456	plant-type cell wall organization	15	0.02116
transferase activity, transferring glycosyl groups	95	0.02526	FKBP-type PPIase family	5	0.02144
response to cold	125	0.02774	PPIase FKBP-type domain	5	0.02144
Senescence Auxin Signaling	16	0.02893	peptidyl-proline modification	5	0.02144
apoplast	151	0.03113	FK506 binding	5	0.02144
Glucosinolate biosynthesis from dihomomethionine	8	0.03148	cytoplasm	1146	0.02333
PRA1 family	9	0.03195	protein polyubiquitination	20	0.02389

Table A.6. Differentially regulated gene sets in spring vs winter at 30 days detected from Gene Set Enrichment Analysis (GSEA) (Continued)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in winter	No. of measured entities	p-value
MATE	11	0.03268	TC 3.D.1.6	16	0.02451
drug transmembrane transporter activity	11	0.03268	response to hydrogen peroxide	23	0.02589
monosaccharide transmembrane transporter activity	5	0.03273	hydrolase activity, acting on ester bonds	16	0.02822
signal transduction	67	0.03509	auxin-activated signaling pathway	36	0.02841
nitrate assimilation	12	0.03532	O-methyltransferase activity	6	0.02867
xyloglucan:xyloglucosyl transferase	12	0.03662	mitochondrial envelope	7	0.03059
xyloglucan:xyloglucosyl transferase	12	0.03662	chitin binding	6	0.03171
xyloglucan:xyloglucosyl transferase	12	0.03662	phosphorylphosphatase	7	0.03271
protein kinase binding	11	0.0371	response to cadmium ion	196	0.03281
response to light stimulus	41	0.03779	response to high light intensity	18	0.03333
hydrogen-transporting ATP synthase, F1 sector	5	0.03825	cellular water homeostasis	12	0.03448
dioxygenase activity	14	0.03896	cellular response to stress	12	0.03448
EamA domain	18	0.03932	glycerol channel activity	12	0.03448
oxylipin biosynthetic process	9	0.03998	electron carrier activity	55	0.03489
secondary metabolic process	5	0.04036	Cold-Stress Signaling	8	0.03591
protein kinase domain	120	0.041	root morphogenesis	6	0.03661
MADS box protein	6	0.0411	ATP synthesis coupled proton transport	11	0.03684
MADS box protein	6	0.0411	response to salt stress	218	0.03702

Table A.6. Differentially regulated gene sets in spring vs winter at 30 days detected from Gene Set Enrichment Analysis (GSEA) (Continued)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in winter	No. of measured entities	p-value
			oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of		
lignin biosynthetic process	22	0.04126	oxygen into both donors	11	0.03703
ATP binding	522	0.04212	glycosyl hydrolase 1 family	10	0.03718
cellulose synthase (UDP-forming) activity	8	0.04371	beta-glucosidase activity	10	0.03718
lipid metabolic process	29	0.04375	flavanone 3-dioxygenase	6	0.03845
metal ion transport	19	0.04378	calmodulin binding	44	0.03908
response to brassinosteroid	9	0.04416	IAAs	7	0.0402
v-SNARE coiled-coil homology domain	7	0.04419	ribosomal large subunit assembly	13	0.04084
longin domain	7	0.04419	regulation of defense response	16	0.04095
growth	10	0.04555	structural constituent of cell wall	5	0.04167
auxin-activated signaling pathway	39	0.04746	negative regulation of programmed cell death	8	0.04256
sulfotransferase 1 family	5	0.04925	cytochrome b5 family	5	0.0436
sulfotransferase	5	0.04925	cellular response to heat	11	0.04383

Table A.6. Differentially regulated gene sets in spring vs winter at 30 days detected from Gene Set Enrichment Analysis (GSEA) (Continued)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in winter	No. of measured entities	p-value
sulfotransferase activity	5	0.04925	ribosomal small subunit assembly	8	0.04392
			double fertilization forming a zygote and endosperm	6	0.04409
			oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	7	0.04559
			ER-associated ubiquitin-dependent protein catabolic process	7	0.04755
			developmental process	6	0.04869
			transcription corepressor activity	6	0.04993

Table A.7. Differentially regulated subnetwork gene sets in spring vs winter at 30 days detected from Sub-network Enrichment Analysis (SNEA)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Winter	No. of measured entities	p-value
Expression Targets of HSF3	8	0.00146	Expression Targets of JAR1	6	0.03456
Expression Targets of STZ	6	0.00669	Expression Targets of RBOHD	6	0.03478
Expression Targets of HSF	11	0.01135	Expression Targets of SIZ1	6	0.04665
Expression Targets of MP	7	0.01185	Binding Partners of HDA6	15	0.00947
Expression Targets of DREB2C	5	0.03068	Binding Partners of WRKY70	5	0.01280
Expression Targets of HSF1	6	0.03543	Binding Partners of SCF(TIR1) complex	5	0.01521
Expression Targets of RBOH F	5	0.0476	Binding Partners of BZR1	14	0.02398
Binding Partners of HSC70-1	9	0.00026	Binding Partners of SGT1B	6	0.02460
Binding Partners of FLS2	15	0.00135	Binding Partners of FLS2	12	0.03677
Binding Partners of CUL3	8	0.01217	Binding Partners of PUB22	5	0.0390
Binding Partners of ABF3	5	0.0254	Binding Partners of TIR1	9	0.03959
Binding Partners of TIR1	6	0.02704	Binding Partners of BRI1	6	0.03999
Binding Partners of HSP70	10	0.03378	Binding Partners of CUL3	8	0.04144
Binding Partners of phytochrome	7	0.04201	Binding Partners of 14-3-3	15	0.044032
Binding Partners of SRFR1	5	0.04273	Binding Partners of bZIP transcription factor	10	0.048842
Binding Partners of SYTA	7	0.04608	Protein Modification Targets of OST1	7	0.008566
Binding Partners of SYP121	8	0.04658	Proteins/Chemicals Regulating Diseases of fungal plant disease	8	0.02828
Protein Modification Targets of CaMK family	16	0.03494	Proteins/Chemicals Regulating Cell Processes of lignification	23	0.003243
Protein Modification Targets of CPK6	6	0.04767	Proteins/Chemicals Regulating Cell Processes of Ca++ export	7	0.007799
Proteins/Chemicals Regulating Diseases of tomato yellow leaf curl	5	0.01579	Proteins/Chemicals Regulating Cell Processes of innate immune response	43	0.011722

Table A.7. Differentially regulated subnetwork gene sets in spring vs winter at 30 days detected from Sub-network Enrichment Analysis (SNEA) (Continued).

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Winter	No. of measured entities	p-value
Proteins/Chemicals Regulating Cell Processes of lignin biosynthesis	20	0.003602	Proteins/Chemicals Regulating Cell Processes of root growth	184	0.018099
Proteins/Chemicals Regulating Cell Processes of seed abscission	9	0.005505	Proteins/Chemicals Regulating Cell Processes of vegetative growth	26	0.020967
Proteins/Chemicals Regulating Cell Processes of photoinhibition	7	0.006022	Proteins/Chemicals Regulating Cell Processes of lipid degradation	6	0.029237
Proteins/Chemicals Regulating Cell Processes of photoprotection	7	0.010112	Proteins/Chemicals Regulating Cell Processes of nitrate uptake	6	0.031259
Proteins/Chemicals Regulating Cell Processes of plant viability	6	0.01061	Proteins/Chemicals Regulating Cell Processes of nuclear membrane fusion	5	0.031492
Proteins/Chemicals Regulating Cell Processes of lignification	31	0.01141	Proteins/Chemicals Regulating Cell Processes of superoxide anion generation	9	0.031698
Proteins/Chemicals Regulating Cell Processes of lignin content	12	0.012972	Proteins/Chemicals Regulating Cell Processes of reductive pentose-phosphate cycle	5	0.034276
Proteins/Chemicals Regulating Cell Processes of leaf size	21	0.019888	Proteins/Chemicals Regulating Cell Processes of somatic embryogenesis	8	0.035136
Proteins/Chemicals Regulating Cell Processes of seed growth	5	0.021204	Proteins/Chemicals Regulating Cell Processes of jasmonate response	27	0.036007
Proteins/Chemicals Regulating Cell Processes of fertilization	23	0.023507	Proteins/Chemicals Regulating Cell Processes of nonphotochemical quenching	7	0.039249
Proteins/Chemicals Regulating Cell Processes of callus development	12	0.023772	Proteins/Chemicals Regulating Cell Processes of stem growth	15	0.044802
Proteins/Chemicals Regulating Cell Processes of lipid peroxidation	17	0.023869	Proteins/Chemicals Regulating Cell Processes of heat-shock response	13	0.046624

Table A.7. Differentially regulated subnetwork gene sets in spring vs winter at 30 days detected from Sub-network Enrichment Analysis (SNEA) (Continued).

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Winter	No. of measured entities	p-value
Proteins/Chemicals Regulating Cell Processes of xylem loading	11	0.03033	Upstream Neighbors of Ca2+	20	0.000781
Proteins/Chemicals Regulating Cell Processes of negative gravitropism	5	0.031428	Upstream Neighbors of COI1	11	0.00274
Proteins/Chemicals Regulating Cell Processes of root differentiation	28	0.032618	Upstream Neighbors of lignification	23	0.002832
Proteins/Chemicals Regulating Cell Processes of secondary metabolism	5	0.035186	Upstream Neighbors of CO2	6	0.00548
Proteins/Chemicals Regulating Cell Processes of meristem initiation	13	0.035346	Upstream Neighbors of auxin response factor	11	0.005713
Proteins/Chemicals Regulating Cell Processes of heat-shock response	19	0.035644	Upstream Neighbors of IAA7	6	0.005939
Proteins/Chemicals Regulating Cell Processes of membrane depolarization	5	0.037249	Upstream Neighbors of Ca++ export	7	0.007337
Proteins/Chemicals Regulating Cell Processes of photosynthetic electron transport	6	0.040296	Upstream Neighbors of RRTF1	5	0.009431
Proteins/Chemicals Regulating Cell Processes of sugar concentration	8	0.042553	Upstream Neighbors of innate immune response	43	0.00969
Proteins/Chemicals Regulating Cell Processes of xylem development	14	0.044	Upstream Neighbors of UDP-glucose	6	0.010892
Upstream Neighbors of heat shock protein	18	0.00012	Upstream Neighbors of root growth	184	0.012684
Upstream Neighbors of lignin	21	0.001133	Upstream Neighbors of superoxide dismutase	19	0.014166
Upstream Neighbors of lignin biosynthesis	20	0.002927	Upstream Neighbors of phosphatidylethanolamine	5	0.014245

Table A.7. Differentially regulated subnetwork gene sets in spring vs winter at 30 days detected from Sub-network Enrichment Analysis (SNEA) (Continued).

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Winter	No. of measured entities	p-value
Upstream Neighbors of seed abscission	9	0.004799	Upstream Neighbors of alpha-amylase	11	0.017035
Upstream Neighbors of photoinhibition	7	0.005444	Upstream Neighbors of vegetative growth	26	0.019124
Upstream Neighbors of monosaccharide	7	0.006822	Upstream Neighbors of IAA5	5	0.021059
Upstream Neighbors of HSF	13	0.008886	Upstream Neighbors of GA20OX1	10	0.02457
Upstream Neighbors of linoleic acid	5	0.008943	Upstream Neighbors of TOR	5	0.024631
Upstream Neighbors of HSFA3	7	0.009291	Upstream Neighbors of fungal plant disease	8	0.026126
Upstream Neighbors of photoprotection	7	0.009512	Upstream Neighbors of WOX5	5	0.026867
Upstream Neighbors of lignification	31	0.009808	Upstream Neighbors of ATP	32	0.027197
Upstream Neighbors of plant viability	6	0.010001	Upstream Neighbors of lipid degradation	6	0.027558
Upstream Neighbors of phenylpropanoid	16	0.011595	Upstream Neighbors of DREB1A	25	0.029392
Upstream Neighbors of lignin content	12	0.011703	Upstream Neighbors of superoxide anion generation	9	0.02965
Upstream Neighbors of cellulose	11	0.012655	Upstream Neighbors of nuclear membrane fusion	5	0.029968
Upstream Neighbors of photosystem II reaction center	27	0.012759	Upstream Neighbors of ADP	13	0.030419
Upstream Neighbors of HSF1	6	0.013888	Upstream Neighbors of nitrate uptake	6	0.030593
Upstream Neighbors of indole-3-acetic acid	29	0.014903	Upstream Neighbors of polyamine	12	0.031171
Upstream Neighbors of spermidine	7	0.015634	Upstream Neighbors of H+-transporting two-sector ATPase	16	0.031523
Upstream Neighbors of KIN1	10	0.016262	Upstream Neighbors of jasmonate response	27	0.032613

Table A.7. Differentially regulated subnetwork gene sets in spring vs winter at 30 days detected from Sub-network Enrichment Analysis (SNEA) (Continued).

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Winter	No. of measured entities	p-value
Upstream Neighbors of leaf size	21	0.018463	Upstream Neighbors of reductive pentose-phosphate cycle	5	0.033263
Upstream Neighbors of seed growth	5	0.019617	Upstream Neighbors of somatic embryogenesis	8	0.033592
Upstream Neighbors of fertilization	23	0.020252	Upstream Neighbors of pyruvate decarboxylase	5	0.034571
Upstream Neighbors of tomato yellow leaf curl	5	0.020515	Upstream Neighbors of RPM1	5	0.035089
Upstream Neighbors of lipid peroxidation	17	0.021442	Upstream Neighbors of CBF1	17	0.035714
Upstream Neighbors of callus development	12	0.021474	Upstream Neighbors of NO3- transporter	8	0.03677
Upstream Neighbors of flavonols	7	0.026461	Upstream Neighbors of nonphotochemical quenching	7	0.037921
Upstream Neighbors of xylem loading	11	0.028535	Upstream Neighbors of PAP1	10	0.03794
Upstream Neighbors of root differentiation	28	0.028684	Upstream Neighbors of stem growth	15	0.039544
Upstream Neighbors of spermine	8	0.02881	Upstream Neighbors of lignin	22	0.042064
Upstream Neighbors of pipecolic acid	5	0.029538	Upstream Neighbors of heat-shock response	13	0.042797
Upstream Neighbors of ribosome protein	5	0.029585	Upstream Neighbors of CAT2	8	0.043315
Upstream Neighbors of negative gravitropism	5	0.030471	Upstream Neighbors of IAAs	15	0.044662
Upstream Neighbors of monolignol	7	0.030576	Upstream Neighbors of cell death	147	0.045226

Table A.7. Differentially regulated subnetwork gene sets in spring vs winter at 30 days detected from Sub-network Enrichment Analysis (SNEA) (Continued).

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Winter	No. of measured entities	p-value
Upstream Neighbors of heat-shock response	19	0.03217	Upstream Neighbors of response to oxidative stress	12	0.048452
Upstream Neighbors of meristem initiation	13	0.032689	Upstream Neighbors of shoot morphogenesis	6	0.048753
Upstream Neighbors of NIR1	5	0.033078	Upstream Neighbors of AXR1	9	0.049112
Upstream Neighbors of secondary metabolism	5	0.033098	Upstream Neighbors of GSH2	6	0.04984
Upstream Neighbors of ABF1	5	0.033907			
Upstream Neighbors of membrane depolarization	5	0.035553			
Upstream Neighbors of PAD3	8	0.037135			
Upstream Neighbors of photosynthetic electron transport	6	0.037735			
Upstream Neighbors of OPR3	5	0.037772			
Upstream Neighbors of xylem development	14	0.039082			
Upstream Neighbors of caffeoyl-CoA	5	0.039639			
Upstream Neighbors of sugar concentration	8	0.039662			
Upstream Neighbors of glucose	12	0.040144			
Upstream Neighbors of fructose	7	0.04058			
Upstream Neighbors of Ca2+	31	0.041904			
Upstream Neighbors of immune response	70	0.042575			

Table A.7. Differentially regulated subnetwork gene sets in spring vs winter at 30 days detected from Sub-network Enrichment Analysis (SNEA) (Continued).

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Winter	No. of measured entities	p-value
Upstream Neighbors of phenylalanine ammonia-lyase	10	0.044963			
Upstream Neighbors of WRKY33	17	0.045339			
Upstream Neighbors of polyamine	13	0.045825			
Upstream Neighbors of carotenoid	11	0.046753			
Upstream Neighbors of EREBP	8	0.048094			
Upstream Neighbors of SEP3	6	0.048504			
Upstream Neighbors of RBOH F	5	0.049407			

Table A.8. Differentially regulated gene sets in spring vs winter at 60 days detected from Gene Set Enrichment Analysis (GSEA)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Winter	No. of measured entities	p-value
3-chloroallyl aldehyde dehydrogenase activity	5	0.01554	abscisic acid binding	5	0.01599
aging	12	0.01385	abscisic acid receptor	5	0.01914
aldehyde dehydrogenase (NAD) activity	5	0.01554	amino acid transmembrane transporter activity	16	0.02273
aldehyde dehydrogenase family	5	0.01554	apoplast	157	0.02924
amino acid transmembrane transport	8	0.03505	basipetal auxin transport	5	0.04947
amino acid transmembrane transporter activity	16	0.00275	calcium-dependent phospholipid binding	5	0.04007
amino acid transport	14	0.00428	calcium-mediated signaling	8	0.02509
AMP salvage	5	0.00112	CBF/NF-Y transcription factors	9	0.03665
apoplast	150	0.00027	cell wall	182	6.23E-05
ARR-A type family	5	0.00366	cell wall biogenesis	16	0.0207
beta-fructofuranosidase	6	0.03329	cellular copper ion homeostasis	5	0.02253
calcium ion binding	62	0.04074	cellular heat acclimation	5	0.021
camalexin biosynthetic process	6	0.00387	cellular response to heat	12	0.00472
carbohydrate metabolic process	67	0.00341	cellular response to hypoxia	7	0.00126
carboxylic ester hydrolase activity	28	0.01407	cellular response to UV-B	5	0.03911
carboxylic ester hydrolase superfamily	18	0.00648	chaperone-mediated protein folding	8	0.03276
cell wall	175	3.9E-07	Cold-Stress Signaling	7	0.03732
cellular amino acid metabolic process	14	0.03378	copper ion binding	99	0.00924
cellular response to ethylene stimulus	5	0.02214	cytoplasmic stress granule	6	0.0089

Table A.8. Differentially regulated gene sets in spring vs winter at 60 days detected from Gene Set Enrichment Analysis (GSEA) (Continued)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Winter	No. of measured entities	p-value
cellular response to phosphate starvation	18	0.04923	DREB	5	0.02918
cellular response to stress	11	0.01973	drought recovery	6	0.01516
cellular water homeostasis	11	0.01973	E2	24	0.02433
chitin binding	7	0.00747	endonuclease activity	6	0.04983
chitin catabolic process	7	0.00429	extracellular region	393	3.61E-05
chitinase activity	6	0.01091	FK506 binding	6	0.01069
chloroplast thylakoid lumen	9	0.02793	FKBP-type PPIase family	5	0.01431
circadian rhythm	25	0.02997	glutathione metabolic process	29	0.02188
copper ion binding	90	0.03615	glutathione transferase	29	0.00615
cytokinin-activated signaling pathway	17	0.01774	glutathione transferase activity	30	0.00509
Cytokinins Signaling	14	0.00244	glycerol metabolic process	7	0.01867
cytoplasm	1021	0.04066	GST C-terminal domain	32	0.0008
cytosolic ribosome	104	0.04341	GST N-terminal domain	30	0.00279
defense response by callose deposition in cell wall	10	0.02404	heat acclimation	14	0.04143
defense response to oomycetes	9	0.00637	heme binding	79	0.00106
esterase	5	0.04351	hydrogen peroxide catabolic process	29	0.00019
expansin	7	0.01282	hydrogen-transporting ATP synthase, F1 sector	5	0.04403
extracellular region	339	4.1E-07	hydrolase activity, acting on glycosyl bonds	12	0.02049

Table A.8. Differentially regulated gene sets in spring vs winter at 60 days detected from Gene Set Enrichment Analysis (GSEA) (Continued)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Winter	No. of measured entities	p-value
FAD-binding FR-type domain	5	0.02184	hydrolase activity, hydrolyzing O-glycosyl compounds	31	0.0468
Fe2OG dioxygenase domain	10	0.03759	indole glucosinolate metabolic process	7	0.03468
G2-like family	10	0.03707	inorganic diphosphatase	5	0.01567
gibberellic acid mediated signaling pathway	11	0.00424	inorganic diphosphatase activity	6	0.00921
Gibberellin Signaling	13	0.01423	lactoperoxidase	24	0.00026
glucan endo-1,3-beta-D-glucosidase	5	0.01926	lactoperoxidase	24	0.00026
glucan endo-1,3-beta-D-glucosidase activity	5	0.01926	lactoperoxidase	24	0.00026
Glucosinolate biosynthesis from dihomomethionine	11	0.00343	lactoperoxidase	24	0.00026
Glucosinolate biosynthesis from homomethionine	5	0.04343	large ribosomal subunit	18	0.04332
glucosinolate biosynthetic process	12	0.00323	LIM domain	7	0.02414
glucosinolate catabolic process	8	0.03153	lipase activity	7	0.03099
glycerol channel activity	11	0.01973	lipid particle	5	0.01745
heme binding	63	0.01569	manganese ion binding	11	0.00677
hydrogen peroxide catabolic process	19	0.0208	metal ion transport	20	0.04736
hydrolase activity, acting on ester bonds	28	0.00411	mitochondrial proton-transporting ATP synthase complex, catalytic core F(1)	6	0.02097
hydrolase activity, hydrolyzing O-glycosyl compounds	38	0.0012	NAC family	25	0.02718

Table A.8. Differentially regulated gene sets in spring vs winter at 60 days detected from Gene Set Enrichment Analysis (GSEA) (Continued)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Winter	No. of measured entities	p-value
induced systemic resistance	5	0.00562	nuclease activity	5	0.00587
killing of cells of other organism	8	0.01319	nutrient reservoir activity	13	0.01727
leaf development	28	0.02256	CBF/NF-Y transcription factors	5	0.03941
lignin biosynthetic process	23	0.03439	oxalate decarboxylase activity	5	0.03763
lipid catabolic process	27	0.00093	oxalate metabolic process	5	0.03763
L-phenylalanine catabolic process	5	0.03473	oxidation-reduction process	368	0.00146
			oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of		
L-serine biosynthetic process	5	0.04208	oxygen into both donors	12	0.02722
MADS box protein	7	0.01787	P4HA family	5	0.02507
MADS box protein	7	0.01787	P4HA family	5	0.02507
magnesium ion binding	39	0.03836	peptidyl-proline hydroxylation to 4-hydroxy-L-proline	5	0.02507
metal ion transport	18	0.03308	peptidyl-proline modification	6	0.01069
methylation	48	0.01535	peroxidase activity	40	0.00205
negative regulation of seed germination	6	0.04228	phosphorylphosphatase	8	0.0086
nitrate assimilation	8	0.01049	plant-type cell wall organization	13	0.01436
nutrient reservoir activity	10	0.00518	PPIase FKBP-type domain	5	0.01431
oligopeptide transporter	7	0.00868	PPR (pentatricopeptide) repeat	9	0.01775
oligopeptide transporter	7	0.00868	procollagen-proline 4-dioxygenase activity	5	0.02507

Table A.8. Differentially regulated gene sets in spring vs winter at 60 days detected from Gene Set Enrichment Analysis (GSEA) (Continued)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Winter	No. of measured entities	p-value
O-methyltransferase activity	6	0.00154	procollagen-proline dioxygenase	5	0.02507
oxidation-reduction process	300	0.0063	procollagen-proline dioxygenase	5	0.02507
phenylpropanoid biosynthetic process	7	0.04098	procollagen-proline dioxygenase	5	0.02507
phosphorelay response regulator activity	10	0.00812	protein polyubiquitination	21	0.022
phosphorelay sensor kinase activity	5	0.04351	proton transport	11	0.04965
phosphorelay signal transduction system	14	0.0082	removal of superoxide radicals	7	0.02463
photosynthesis	22	0.02394	response to anoxia	5	0.00863
plant-type cell wall	81	0.00072	response to chitin	71	0.04194
plant-type cell wall loosening	8	0.04444	response to cold	116	0.01561
plant-type cell wall organization	12	0.0099	response to heat	83	4.21E-05
plasmodesma	370	0.00394	response to high light intensity	26	0.00039
poly(U) RNA binding	8	0.0216	response to hydrogen peroxide	28	0.00197
polysaccharide catabolic process	5	0.00605	response to hypoxia	23	0.02756
primary root development	11	0.01615	response to nitrate	13	0.02343
protein import into nucleus	10	0.02943	response to oxidative stress	126	0.00017
protein kinase binding	10	0.04574	response to temperature stimulus	8	0.00773
protein-chromophore linkage	7	0.04201	response to toxic substance	32	0.03973
response to abscisic acid	121	0.02991	response to water deprivation	108	0.00991
response to cold	110	0.04125	response to wounding	80	0.04259
response to cytokinin	82	0.03372	ribosomal small subunit assembly	5	0.0388

Table A.8. Differentially regulated gene sets in spring vs winter at 60 days detected from Gene Set Enrichment Analysis (GSEA) (Continued)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Winter	No. of measured entities	p-value
response to gibberellin	26	0.00066	secondary metabolite biosynthetic process	15	0.03971
response to insect	6	0.02752	small heat shock protein	13	0.0004
response to jasmonic acid	58	0.02126	small heat shock protein	13	0.0004
response to light stimulus	42	0.02898	superoxide dismutase	5	0.04438
response to osmotic stress	41	0.04549	superoxide dismutase activity	5	0.04438
response to other organism	14	0.01897	toxin catabolic process	27	0.00761
response to water deprivation	90	0.00112	translational elongation	25	0.00495
seed coat development	5	0.04381	ubiquinol-cytochrome-c reductase activity	9	0.02274
SNAP receptor activity	17	0.01909	ubiquitin-conjugating enzyme family	28	0.01907
SNARE binding	15	0.03603	ubiquitin-protein ligase	23	0.01839
sucrose alpha-glucosidase activity	6	0.03329	xyloglucan:xyloglucosyl transferase	10	0.00261
sucrose metabolic process	6	0.02829	xyloglucan:xyloglucosyl transferase	10	0.00261
symporter activity	16	0.02338	xyloglucan:xyloglucosyl transferase	10	0.00261
systemic acquired resistance	9	0.00775	xyloglucan:xyloglucosyl transferase activity	10	0.00261
translational elongation	33	0.04435			
transport	62	0.00495			
tryptophan biosynthetic process	10	0.02329			
water channel activity	12	0.01501			
water transport	9	0.0145			
xyloglucan metabolic process	15	0.02001			
xyloglucan:xyloglucosyl transferase	10	0.00218			
xyloglucan:xyloglucosyl transferase	10	0.00218			

Table A.8. Differentially regulated gene sets in spring vs winter at 60 days detected from Gene Set Enrichment Analysis (GSEA) (Continued)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Winter	No. of measured entities	p-value
xyloglucan:xyloglucosyl transferase	10	0.00218			
xyloglucan:xyloglucosyl transferase activity	11	0.00916			

Table A.9. Differentially regulated subnetwork gene sets in spring vs winter at 60days detected from Sub-network Enrichment Analysis (SNEA)

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Spring	No. of measured entities	p-value
Expression Targets of HPY2	7	0.01581485	Expression Targets of RBOH F	5	0.01313
Expression Targets of MYB51	6	0.01714367	Expression Targets of SIZ1	8	0.0133
Expression Targets of MYB34	9	0.02525484	Expression Targets of BES1	8	0.01514
Expression Targets of JAR1	7	0.02983109	Expression Targets of HSFA2	5	0.04245
Expression Targets of RGA1	10	0.04068884	Expression Targets of ABI4	8	0.04518
Expression Targets of ABI4	7	0.04858455	Expression Targets of HSF	12	0.04801
Binding Partners of AHP2	9	0.00370184	Binding Partners of CaMK family	5	0.00455
Binding Partners of FLS2	14	0.00618697	Binding Partners of RGA1	9	0.01412
Binding Partners of protein phosphatase	5	0.00815112	Binding Partners of CBL10	5	0.02007
Binding Partners of AHP3	5	0.0122712	Binding Partners of SGT1B	5	0.0228
Binding Partners of BZR1	16	0.01345681	Binding Partners of 14-3-3	17	0.04521
Binding Partners of AP2	5	0.02146639	Protein Modification Targets of OST1	8	0.03629
Binding Partners of GAI	8	0.02281919	Protein Modification Targets of CUL4	5	0.04388
Binding Partners of SAP18	5	0.02454164	Proteins/Chemicals Regulating Cell Processes of oxidative stress	115	0.00021
Binding Partners of ABI2	5	0.02747693	Proteins/Chemicals Regulating Cell Processes of innate immune response	47	0.00266
Binding Partners of heat shock protein	6	0.02905358	Proteins/Chemicals Regulating Cell Processes of plant defense	146	0.00268
Binding Partners of phytochrome	7	0.03670766	Proteins/Chemicals Regulating Cell Processes of cell death	174	0.00475

Table A.9. Differentially regulated subnetwork gene sets in spring vs winter at 60days detected from Sub-network Enrichment Analysis (SNEA) (Continue).

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Spring	No. of measured entities	p-value
Binding Partners of 14-3-3	14	0.04782817	Proteins/Chemicals Regulating Cell Processes of heat tolerance	58	0.00615
Protein Modification Targets of AHP2	5	0.02049401	Proteins/Chemicals Regulating Cell Processes of lipid metabolism	20	0.00615
Protein Modification Targets of OST1	5	0.04652054	Proteins/Chemicals Regulating Cell Processes of response to osmotic stress	36	0.00893
Proteins/Chemicals Regulating Cell Processes of plant height	22	0.00066552	Proteins/Chemicals Regulating Cell Processes of drought tolerance	79	0.01936
Proteins/Chemicals Regulating Cell Processes of shoot formation	8	0.00163212	Proteins/Chemicals Regulating Cell Processes of defense response	211	0.02024
Proteins/Chemicals Regulating Cell Processes of response to cytokinin stimulus	39	0.00440326	Proteins/Chemicals Regulating Cell Processes of nuclear membrane fusion	5	0.0207
Proteins/Chemicals Regulating Cell Processes of glucosinolate biosynthesis	27	0.00583728	Proteins/Chemicals Regulating Cell Processes of heat-shock response	16	0.02107
Proteins/Chemicals Regulating Cell Processes of lignin content	10	0.00645759	Proteins/Chemicals Regulating Cell Processes of lignification	26	0.02321
Proteins/Chemicals Regulating Cell Processes of photodamage	5	0.00971786	Proteins/Chemicals Regulating Cell Processes of detoxification (process)	61	0.02335
Proteins/Chemicals Regulating Cell Processes of meristem initiation	11	0.01331132	Proteins/Chemicals Regulating Cell Processes of ROS generation	85	0.0319
Proteins/Chemicals Regulating Cell Processes of leaf size	20	0.01334871	Proteins/Chemicals Regulating Cell Processes of Tricarboxylic acid cycle	17	0.03214

Table A.9. Differentially regulated subnetwork gene sets in spring vs winter at 60days detected from Sub-network Enrichment Analysis (SNEA) (Continued).

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Spring	No. of measured entities	p-value
Proteins/Chemicals Regulating Cell Processes of photosynthetic electron transport	5	0.01600552	Proteins/Chemicals Regulating Cell Processes of somatic embryogenesis	12	0.03694
Proteins/Chemicals Regulating Cell Processes of photoinhibition	6	0.01700221	Proteins/Chemicals Regulating Cell Processes of leaf area	6	0.03763
Proteins/Chemicals Regulating Cell Processes of response to phosphate starvation	5	0.01700301	Proteins/Chemicals Regulating Cell Processes of seed germination	216	0.04039
Proteins/Chemicals Regulating Cell Processes of sugar concentration	9	0.01780858	Proteins/Chemicals Regulating Cell Processes of apoptosis	118	0.04556
Proteins/Chemicals Regulating Cell Processes of superoxide anion generation	7	0.01919256	Proteins/Chemicals Regulating Cell Processes of disease resistance	110	0.0459
Proteins/Chemicals Regulating Cell Processes of seed dormancy	20	0.02417977	Proteins/Chemicals Regulating Cell Processes of endoplasmic reticulum stress	15	0.04735
Proteins/Chemicals Regulating Cell Processes of stem growth	19	0.02662444	Upstream Neighbors of oxidative stress	115	0.00023
Proteins/Chemicals Regulating Cell Processes of lateral root number	6	0.02738092	Upstream Neighbors of innate immune response	47	0.00285
Proteins/Chemicals Regulating Cell Processes of shoot regeneration	11	0.0274627	Upstream Neighbors of plant defense	146	0.00303
Proteins/Chemicals Regulating Cell Processes of leaf yield	5	0.02747798	Upstream Neighbors of cell death	174	0.00553
Proteins/Chemicals Regulating Cell Processes of xylem development	14	0.0314731	Upstream Neighbors of caspase	7	0.00621

Table A.9. Differentially regulated subnetwork gene sets in spring vs winter at 60days detected from Sub-network Enrichment Analysis (SNEA) (Continued).

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Spring	No. of measured entities	p-value
Proteins/Chemicals Regulating Cell Processes of nonphotochemical quenching	7	0.03808961	Upstream Neighbors of lipid metabolism	20	0.00624
Proteins/Chemicals Regulating Cell Processes of negative gravitropism	5	0.03866249	Upstream Neighbors of heat tolerance	58	0.0064
Proteins/Chemicals Regulating Cell Processes of nodulation	19	0.03867594	Upstream Neighbors of heat shock protein	17	0.00737
Proteins/Chemicals Regulating Cell Processes of seed abscission	7	0.04158778	Upstream Neighbors of response to osmotic stress	36	0.00902
Proteins/Chemicals Regulating Cell Processes of root hair tip growth	6	0.0416314	Upstream Neighbors of phenylpropanoid	10	0.01013
Proteins/Chemicals Regulating Cell Processes of meristem size	25	0.04581174	Upstream Neighbors of lignin	20	0.0108
Proteins/Chemicals Regulating Cell Processes of sugar response	18	0.04705212	Upstream Neighbors of VSP2	15	0.01147
Proteins/Chemicals Regulating Cell Processes of meristem function	40	0.04772572	Upstream Neighbors of ABI4	10	0.01496
Proteins/Chemicals Regulating Cell Processes of lignification	27	0.04926736	Upstream Neighbors of RRTF1	5	0.01675
Upstream Neighbors of plant height	22	0.00076518	Upstream Neighbors of ABI1	16	0.01767
Upstream Neighbors of shoot formation	8	0.00157449	Upstream Neighbors of nuclear membrane fusion	5	0.02066
Upstream Neighbors of RR22	6	0.00244837	Upstream Neighbors of heat-shock response	16	0.0208
Upstream Neighbors of RGA1	14	0.00295035	Upstream Neighbors of drought tolerance	79	0.02102

Table A.9. Differentially regulated subnetwork gene sets in spring vs winter at 60days detected from Sub-network Enrichment Analysis (SNEA) (Continued).

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Spring	No. of measured entities	p-value
Upstream Neighbors of PLT1	10	0.00404466	Upstream Neighbors of COI1	11	0.02117
Upstream Neighbors of RR24	5	0.00414906	Upstream Neighbors of polyamine	14	0.02122
Upstream Neighbors of heat shock protein	16	0.00454308	Upstream Neighbors of CAT1	5	0.02237
Upstream Neighbors of response to cytokinin stimulus	39	0.00550606	Upstream Neighbors of lignification	26	0.02325
Upstream Neighbors of ABF1	5	0.00621533	Upstream Neighbors of superoxide dismutase	20	0.02347
Upstream Neighbors of TIR1	5	0.00685867	Upstream Neighbors of detoxification (process)	61	0.02394
Upstream Neighbors of lignin content	10	0.0069179	Upstream Neighbors of defense response	211	0.02396
Upstream Neighbors of ARR6	8	0.00704095	Upstream Neighbors of WRKY	25	0.02736
Upstream Neighbors of H2O	13	0.00724893	Upstream Neighbors of PP2C	8	0.02935
Upstream Neighbors of glucosinolate biosynthesis	27	0.00745403	Upstream Neighbors of iron superoxide dismutase	5	0.03096
Upstream Neighbors of fructose	6	0.00770338	Upstream Neighbors of UDP-glucose	8	0.03132
Upstream Neighbors of SLY1	5	0.00787117	Upstream Neighbors of beta-galactosidase	9	0.03246
Upstream Neighbors of glycerol	5	0.00985913	Upstream Neighbors of Tricarboxylic acid cycle	17	0.03337
Upstream Neighbors of GEA6	5	0.0098688	Upstream Neighbors of ROS generation	85	0.03575
Upstream Neighbors of photodamage	5	0.01018715	Upstream Neighbors of alpha-linolenic acid	7	0.03657

Table A.9. Differentially regulated subnetwork gene sets in spring vs winter at 60days detected from Sub-network Enrichment Analysis (SNEA) (Continued).

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Spring	No. of measured entities	p-value
Upstream Neighbors of PLT2	6	0.01050237	Upstream Neighbors of RD22	5	0.03727
Upstream Neighbors of ethylene	44	0.01129375	Upstream Neighbors of leaf area	6	0.03757
Upstream Neighbors of CYP79B3	9	0.01288112	Upstream Neighbors of somatic embryogenesis	12	0.0378
Upstream Neighbors of indole-3-acetaldoxime	6	0.01397439	Upstream Neighbors of AXR1	6	0.04076
Upstream Neighbors of meristem initiation	11	0.01447999	Upstream Neighbors of SCR	5	0.04267
Upstream Neighbors of ABF2	9	0.01452798	Upstream Neighbors of WRKY25	7	0.04278
Upstream Neighbors of lignin	20	0.01465841	Upstream Neighbors of unsaturated fatty acid	7	0.04356
Upstream Neighbors of COR47	10	0.01499136	Upstream Neighbors of GSH2	7	0.04459
Upstream Neighbors of leaf size	20	0.01515079	Upstream Neighbors of RBR1	8	0.04701
Upstream Neighbors of ABF	6	0.01623004	Upstream Neighbors of seed germination	216	0.04737
Upstream Neighbors of photosynthetic electron transport	5	0.01739443	Upstream Neighbors of endoplasmic reticulum stress	15	0.04921
Upstream Neighbors of phenylpropanoid	17	0.01765142			
Upstream Neighbors of photoinhibition	6	0.01775196			
Upstream Neighbors of DFR	8	0.01801738			
Upstream Neighbors of response to phosphate starvation	5	0.01826884			
Upstream Neighbors of tryptophan	14	0.01961961			

Table A.9. Differentially regulated subnetwork gene sets in spring vs winter at 60days detected from Sub-network Enrichment Analysis (SNEA) (Continued).

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Spring	No. of measured entities	p-value
Upstream Neighbors of sugar concentration	9	0.01976596			
Upstream Neighbors of BGL2	10	0.02045246			
Upstream Neighbors of superoxide anion generation	7	0.02121305			
Upstream Neighbors of LDOX	7	0.02259226			
Upstream Neighbors of GSH2	5	0.02280026			
Upstream Neighbors of PHYB	10	0.02318342			
Upstream Neighbors of protochlorophyllide	7	0.02785716			
Upstream Neighbors of seed dormancy	20	0.02894936			
Upstream Neighbors of shoot regeneration	11	0.02916167			
Upstream Neighbors of leaf yield	5	0.02959316			
Upstream Neighbors of NRT2:1	7	0.02988875			
Upstream Neighbors of lateral root number	6	0.02995143			
Upstream Neighbors of ABI3	13	0.03095368			
Upstream Neighbors of photosystem II reaction center	31	0.03123743			
Upstream Neighbors of stem growth	19	0.03153891			
Upstream Neighbors of xylem development	14	0.03401411			
Upstream Neighbors of WOX5	8	0.03403919			
Upstream Neighbors of OST1	11	0.03449254			
Upstream Neighbors of CSD1	5	0.0359363			

Table A.9. Differentially regulated subnetwork gene sets in spring vs winter at 60days detected from Sub-network Enrichment Analysis (SNEA) (Continued).

Gene sets Upregulated in Spring	No. of measured entities	p-value	Gene sets Upregulated in Spring	No. of measured entities	p-value
Upstream Neighbors of sulfate	7	0.03612918			
Upstream Neighbors of monolignol	9	0.03666427			
Upstream Neighbors of CYP83B1	6	0.03862085			
Upstream Neighbors of nonphotochemical quenching	7	0.03906974			
Upstream Neighbors of negative gravitropism	5	0.04230431			
Upstream Neighbors of root hair tip growth	6	0.04384365			
Upstream Neighbors of seed abscission	7	0.0438703			
Upstream Neighbors of ribosome protein	5	0.04491745			
Upstream Neighbors of nodulation	19	0.04567303			
Upstream Neighbors of NYE1	7	0.04659086			
Upstream Neighbors of HSF	12	0.04684083			
Upstream Neighbors of RR2	6	0.04781564			
Upstream Neighbors of HB1	5	0.04800838			
Upstream Neighbors of glycine	6	0.04947309			