Differential Expression Framework of Genes Underpinning Root Hairs in Wheat cv. Zincol-16 using Transcriptomics

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By

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This is to certify that the dissertation entitled "**Differential Expression Framework of Genes Underpinning Root Hairs in Wheat cv. Zincol-16 using Transcriptomics**" submitted by **Fatima Saeed** is accepted in its present form by the Department of Plant Sciences, Quaid-i-Azam University Islamabad, Pakistan, as satisfying the dissertation requirement for the degree of Master of Philosophy (M.Phil.) in Botany.

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TO

MY

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Fatima Saeed

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LIST OF ABBREVIATIONS

Gb	Gigabases
IWGSC	International Wheat Genome Sequencing Consortium
FAO	Food and Agriculture Organization
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
GSDS	Gene Structure Display Server
HISAT2	Hierarchical Indexing for Spliced Alignment of Transcripts 2
TMM	Trimmed Mean of M-values
PCA	Principal Component Analysis
CPM	Counts per Million
MDS	Multidimensional Scaling
MD	Mean Difference

ABSTRACT

Root hairs are specialized protrusions of epidermal cells of roots that play a pivotal role in water and nutrient uptake and soil anchorage, making them an intriguing target for investigating diverse aspects of cell biology, cell expansion, differentiation, and physiological. Transcriptomics has emerged as a powerful tool to unravel the intricate molecular mechanisms underlying plant development and responses to environmental cues. Zincol is a Pakistani wheat variety that has garnered attention for its elevated zinc content, holding significant promise for addressing micronutrient deficiencies in South Asia. This study employs RNA sequencing to dissect the transcriptome of Zincol root hairs and roots stripped of root hairs. Root hairs were isolated by gently stirring roots of 4-day old Zincol seedlings in liquid nitrogen and passing the slurry through a metal strainer. One batch of isolated root hairs was used for qRT-PCR and another was sent for RNA sequencing along with stripped roots, total roots and leaf tissue. Through pairwise differential analysis between root hairs and stripped roots, it was found that in root hairs 3,193 genes were upregulated, 7,371 were downregulated and 3.4% genes were exclusively while 9.3% were exclusively expressed in stripped roots. Expression of a gene coding for a C2H2-type domain-containing protein and its homeologues was compared in the four tissues. Expression of several other genes including expansins and xyloglucan endotransglucosylase/hydrolases was also compared. It is important to acknowledge the limitations of this study. The absence of biological replicates in the experimental design introduces certain constraints on the statistical robustness of the findings. Future research endeavors should consider incorporating replicates to strengthen the validity of the results.

CHAPTER 1

INTRODUCTION

1.1. Significance of Wheat

The global population reached 8 billion on 15th of November 2022 and is estimated to reach 9.7 billion by 2050 (DESA, 2022). Because of this growing population, food security will be a major challenge which is why it is one of the sustainable development goals on the 2030 agenda of United Nations (UN, 2015).

There are 250,000 to 300,000 known plant species which are edible for humans. Out of these, 150-200 are cultivated and used by humans. Almost 50% of the crop production worldwide comes from just four crops: sugarcane, maize, wheat, and rice, with cereals being the most traded commodity in terms of quantity (FAO, 2022). Almost 60% of the total calories humans obtain from plants are derived from wheat, rice and maize (FAO, 2004).

Wheat is a staple cereal grown mainly as a food crop on every continent of the earth except Antarctica, contributing 20% of the total calories consumed by humans. It is considered a primary source of energy in many parts of the world because of its carbohydrate content. Its importance is raised by its content of proteins, dietary fiber, vitamin B, minerals like magnesium, zinc and iron and phytochemicals like phenolics and terpenoids. It is considered the richest source of methyl donor betaine. Essential B vitamins especially folates, thiamine, niacin, riboflavin and pyridoxine are found in the bran of wheat grains. Out of these, niacin is of special importance because its deficiency leads to pellagra (Shewry & Hey, 2015) It contains a protein called gluten which gives it its characteristic property of being made into a sticky dough. This sticky dough is then used to make bread, bakery products, noodles and pasta which are consumed globally (Kumar *et al.*, 2011). Wheat is also involved in the production of beer and alcoholic beverages through fermentation, ethanol and biofuel (Cooper, 2015).

1.2. Wheat Production Statistics

Wheat is a staple crop in many regions of the world and is grown on 220 million hectares throughout the world. The global wheat production for 2022 is projected to be 783.8 million tonnes

(FAO, 2022). In Pakistan, wheat is a rabi or a winter crop sown in October or November and harvested in April to May, and is cultivated on 9 million ha. In 2022 its production was 26 million tonnes with 1.9% decrease because of shortage of irrigation water, drought conditions and heat waves in spring. It comprises 1.8% of Pakistan's GDP (GOP, 2022).

In 2021, 77.3% of Pakistan's total wheat imports were from Russia and Ukraine. The armed conflict between these two countries has led to a decline in imports of wheat and other food commodities which has aggravated the food crisis in the global south (GOP, 2022). Another factor that affected Pakistan's food crisis is the severe floods of 2022 which submerged one-third of the country, affecting millions of people, and destroying crops (Qamer *et al.*, 2023). This calls for the expansion of wheat production in developing countries like Pakistan (Khondoker, 2022).

1.3. Origins and Domestication of Wheat

More than 10,000 years ago, human cultures transitioned on a large scale from a lifestyle of hunting and gathering to that of agricultural cultivation and settling. This transition referred to as the Neolithic revolution or the agricultural revolution is when people started domesticating plants like wheat, maize, rice and barley. With the help of biogeographical studies, these crops were associated with regions called centers of origin. The center of origin for crops like wheat and barley was the fertile crescent which stretches from the south-eastern Turkey to modern-day Iraq, Syria, Lebanon, Jordan and Western Iran. Wheat was one of the first cereals that was domesticated and cultivated in this region. The domestication process of plants involves a set of anatomical and morphological changes that occurs in the plants as a result of cultivation and adaptation in environments modified by humans (Charmet, 2011).

The wheat we consume today is the result of interspecific hybridization and polyploidy. *Triticum aestivum* commonly known as bread wheat makes up 95% of the wheat crop grown today. It is the classic example of evolution through allopolyploidization. It is a hexaploid with 21 pairs of chromosomes and 3 sub-genomes. Each sub-genome contains 7 of the 21 pairs. Its genomic constitution is represented by AABBDD; the A genome derived from *Triticum urartu*, B from *Aegilops speltoides* and D from *Aegilops tauschii*.

Around 500,000–150,000 years BP (Before Present), a polyploidization event occurred between two diploid species *T. urartu* and *Ae. speltoides*, forming the tetraploid *Triticum turgidum*

L. ssp dicoccoides which evolved into *T. turgidum ssp dicoccum*, the ancestor of *Triticum durum*, commonly known as the durum wheat. Durum wheat makes up the remaining 5% of the wheat crop grown in the world and it is primarily used to make pasta.

Around 10,000 BP the second polyploidization event occurred between *T. dicoccum* and *A. tauschii*, forming *T. aestivum*, which is found in its domesticated form today (Charmet, 2011; Peterson *et al.*, 2006; Ramírez-González *et al.*, 2018; Dubcovsky *et al.*, 2007). Polyploidy has been said to confer plants the plasticity to be diversified, adapted and domesticated (Salman-Minkov *et al.*, 2017; Leitch & Leitch, 2008). It is known to enlarge cells, alter physiological and growth traits and increase the ability of plants to tolerate stress (Balao *et al.*, 2011).

1.4. Wheat Genomics

Triticum aestivum is a hexaploid (AABBDD = 6x = 2n = 42 chromosomes) with three sub-genomes (A, B and D), each containing 7 homologous pairs of chromosomes. Each sub-genome consists of highly similar genes called homeologues. The total size of its genome is ~17 Gb (giga-base) with each sub-genome accounting for 5.5 Gb. This makes it one of the largest genomes in higher plants, forty times larger than rice. Almost 80% of the genome is comprised of repeats and transposable elements (Choulet *et al*, 2014; Choulet *et al*, 2010). Because of the large size and high repeatability, the progress in wheat genomes and assembling of reference sequences had fallen behind compared to cereals like rice and maize, but the advances in sequencing and assembly technologies have helped resolve these difficulties (Uauy, 2017).

Triticum is a botanical genus that belongs to the tribe Triticeae within the subfamily of Pooideae of the family Poaceae. The genus *Triticum* has species at various ploidy levels, some of which are found in wild, and some exist only in cultivation. The diploid species *T. urartu* is found in the wild while *T. monococcum* exists in both wild and in cultivation. Similarly, the tetraploid species of *Triticum* also have both wild and cultivated forms. On the other hand, the hexaploid species exist only in cultivation (Ortiz *et al*, 2008).

1.5. Challenges in Crop Breeding

In the 20th century people went from using simple selection of crops to plant breeding using scientific principles like those based on Mendel's laws of inheritance which were published in 1866 and rediscovered in 1900. In the 1960s, the food security in the developing world was in a

dire state because the food production couldn't keep up with the growing populations, and the tall and high yield varieties of wheat and rice would lodge or fall over because of the weight of the grains, resulting in significant yield loss. To deal with this problem Dr. Norman Borloug and others introduced semi-dwarf varieties of these crops that didn't lodge, increased grain production and prevented a disaster. The use of disease resistant, semi-dwarf, high yield varieties and the application of fertilizers, herbicides and pesticides lead to staggering doubled up yield of wheat in Pakistan and India (Curtis & Halford, 2014; Hedden, 2003), and this whole period is known as the green revolution.

The downside to the growth in green revolution was the severe environmental impacts of the production, transportation and application of mineral fertilizers. These include the emissions of greenhouse gases, subsequent global warming and eutrophication (Good & Beatty, 2011).

Around the world, many regions are facing the impacts of climate change and global warming which include droughts, intense heat waves, floods, wildfires and rising sea levels. This puts food security at risk especially in the global south. According to the IPCC assessments, the impact of climate change on wheat yields will be -1.3% and crop yields in warmer regions will be disproportionately impacted (Pörtner *et al.*, 2022). In wheat any positive effects of high carbon dioxide levels on yield are negated by rising temperatures (Hasegawa *et al.*, 2022). Unless we reduce our carbon dioxide and greenhouse gas emissions significantly, the global warming of 1.5 C and 2 C will go beyond. The past and the impending emissions have caused many changes which will not be reversible for at least centuries (Pörtner *et al.*, 2022).

In the 21st century, a transition is happening from the green revolution to a second green revolution or an 'Evergreen revolution' in which yields and quality of crops will be improved, and the required inputs like fertilizers, irrigation, pesticides, herbicides, fungicides etc. will be reduced. This is possible by using integrated nutrient management and employment of germplasm that is adapted to grow and yield well in less fertile soils. This will help reduce the environmental impact of intense fertilization (White *et al.*, 2013; Lynch, 2007).

As a major crop produced in and adapted to many regions across the world with varying environments, wheat has diverse genotypic and phenotypic characteristics which enable it to grow and adapt to these environments. By selecting desirable characteristics, we can make cultivars that have a better yield, show resistance to diseases, use less water and fertilizers, and can be adapted

to warmer climates (Rasheed *et al.*, 2016; Mujeeb-Kazi *et al.*, 2013; Hyles *et al.*, 2020). To improve productivity and yield of crops that can grow in nutrient and water poor soils, we can try to manipulate traits of the root system architecture since it is involved in acquiring resources from the soil (Rongsawat *et al.*, 2021). For that to be possible we need to understand the root traits and their variability, and identify traits or phenes that contribute to making the root system more efficient (Lynch & Brown, 2012). Roots can sense stresses like drought and send signals to other parts of the plant. Another important underground part of plants is root hairs which make up about 70% of the root's surface area and helps it penetrate dry soils (Paez-Garcia *et al.*, 2015).

1.6. Significance of Root Hairs

Roots evolved twice during the Devonian period (Pires & Dolan, 2012) while root hairs evolved 400 million years ago (Raven & Edwards, 2001). Terrestrial plants have evolved root systems that are adapted to obtaining sufficient mineral nutrients from soil even when there is a deficiency of said nutrients. The factors that are involved in roots acquiring nutrients from soil include the architecture of root system, the morphological and anatomical properties of root, the rate of uptake of nutrient solution, the symbiotic associations with mycorrhizae and root hairs. For the roots to uptake the nutrient solution, they need to have appropriate morphology to be distributed throughout the soil and be in contact with the nutrients. This is why terrestrial plants have evolved root systems with long roots of smaller diameter, lateral branching and root hairs, which makes their surface area larger (Jungk, 2001).

Root hairs are cylindrical elongations or outgrowths that emanate from the epidermal cells of roots. Epidermal cells that differentiate into root hair cells are called trichoblasts or H cells and those that don't are called atrichoblasts or N cells. This determination depends on position dependent signals that come from the cortical cells lying behind the epidermal cells (Balcerowicz *et al.*, 2015). A root hair has apical, vacuolar and basal zones (Volgger *et al.*, 2010).

Root hairs increase the surface area of root available for water and nutrient acquisition (Datta *et al.*, 2011). Their development occurs in four steps: specification of an epidermal cell into a root hair cell, initiation, tip growth and maturation. Because of these steps, root hairs are used as a model system to study cell specification and polarity (Gilroy & Jones, 2000; Samaj & Menzel, 2004). They are also useful for studying cell expansion because of the high rate of growth i.e., 1

micrometer per minute. Since they are not essential for the viability of a plant, all kinds of mutants with and without root hairs can be studied and analyzed (Grierson *et al.*, 2014).

Root hairs are important for nutrient and water absorption and soil anchorage. Comparisons of root hairless mutants of Arabidopsis to wildtype individuals, shows that the lack of root hairs decreases the mutant's ability to tolerate drought stress, heat and salt stress because they are not able to absorb more water (Tanaka *et al.*, 2014). Such root hairless mutants have also been found and studied in Barley, including *bald root barley* or *brb* which is affected by low phosphorus availability (Gahoonia *et al.*, 2001; Gahoonia & Nielson, 2003) and rhl1.a, rhl1.b and rhl1.c (Chmielewska *et al.*, 2014). Root hairs have a significant role in the absorption of nutrients like phosphorus that are sparingly soluble in soil solution (Leitner *et al.*, 2010). Much of the research done on root hairs is focused on phosphorus acquisition but other elements like nitrogen, iron, zinc and manganese have also been shown to regulate the growth of root hairs (Wang *et al.*, 2016).

As root hairs develop and expand the surface area of roots, they increase the phosphorus absorption (Brown *et al.* 2012). Their geometrical scale also helps them access immobile nutrients (George *et al.*, 2014). Rhizosheath, which is composed of aggregates of soil in the rhizosphere (soil surrounding the roots) bound to the surface of roots (Koebernick *et al.*, 2017), is rich in microbes and mycorrhiza. This increase in interaction between roots and soil facilitates the phosphorus absorption further (Ruiz *et al.*, 2020). All of these factors lead to more than half of the total phosphorus acquired by the roots being accounted for by root hairs (Keyes *et al.*, 2013; Brown *et al.* 2012).

In wheat the rhizosheath is correlated with root hair length and plants with larger rhizosheaths have larger shoot biomasses (James *et al.*, 2016; Delhaize *et al.*, 2015). Longer root hairs uptake phosphorus better than their shorter counterparts (Liu *et al.*, 2017).

1.7. Root Hair Traits and their Genomic Underpinnings

Many genes responsible for the development of root hairs have been identified such as *ROOT HAIR DEFECTIVE SIX-LIKE* or *RSL*. Basic helix-loop-helix (bHLH) transcription factors of *RSL* class I are involved in the development of rhizoids in liverworts and mosses and root hairs in angiosperms (Proust *et al.*, 2016). In Brachypodium distachyon *RSL* class I transcription factors transform cells into root hair cells (Kim & Dolan, 2016). *RSL1* and *RHD6* are involved in targeting

genes that encode bHLH transcription factors like rsl2, rsl3, rsl4 and lrl3 (Bruex *et al.*, 2012; Yi *et al.*, 2010)

The determination of whether an epidermal cell will develop into a root hair cell (trichoblast) or not (atrichoblast) depends on complex activity of transcriptional factors that move from cell to cell. This process is initiated by positional signals that are stronger in future root hair cells in which the expression of *WEREWOLF (WER)* is repressed (Grebe, 2012; Lan *et al.*, 2013).

RHT1 and *RHT2* are responsible for elongating root hairs in maize (Hochholdinger & Tuberosa, 2009). Another study in maize shows that root hair length and density are controlled by roothairless5 or rth5 (Nestler *et al.*, 2014).

A study on Arabidopsis shows that for root hair elongation, the expression of *RSL4* is required. The duration of its expression in the cell determines the length of root hair. *RSL4* transcriptionally regulates other genes which work on elongating root hairs. These genes include *SUPPRESSOR OF ACTIN (SAC1), EXOCSYT SUBUNIT 70A1 (EXO70A1), PEROXIDASE7 (PRX7)* and *CALCIUM-DEPENDENT PROTEIN KINASE11 (CPK11)* (Vijayakumar *et al.*, 2016). The duration of RSL2's presence also has a correlation to the length of root hair (Yi *et al.*, 2010).

In wheat, the variation in the length of root hairs has also been linked to the transcriptional expression of the gene *ROOT HAIR DEFECTIVE6-LIKE 4* or *TaRSL4*. Furthermore, its homeologue from the A genome shows higher expression resulting in longer root hairs (Han *et al.*, 2016). *RSL4* could be regulating growth of root hairs by modifying the cell wall composition as evidenced by the differential expression of cell wall modifying genes in rsl4 mutants (Yi *et al.*, 2010). *EXPA7* and *EXPA18* are in charge of modifying cell walls and are also essential for the formation of root hairs (Cho & Cosgrove, 2002).

During the beginning of elongation of root hairs, *RSL4* is expressed in a pulse. The root hair keeps elongating until *RSL4* is proteolyzed. This production of *RSL4* is a response of root hairs to low phosphate in their environment. This demonstrates how low phosphate induces the synthesis of *RSL4* and therefore the length of root hairs (Datta *et al.*, 2015).

1.8.Root hairs and Phosphorus Acquisition

Crop productivity is limited by the availability of nutrients and water which has led to the development of agricultural systems that are responsible for environmental degradation (Mueller *et al.*, 2012). Phosphorus is an essential mineral nutrient and element of fertilizers, required for the development and reproduction of plants. It is assimilated by plants in the form of inorganic phosphate or orthophosphate (Pi). Generally, in soils the concentration of inorganic phosphate is not optimal for effective growth and productivity, but when it is added to the soil as fertilizer, it either reacts with cations like calcium, magnesium, iron and aluminum or is fixed by microbial species, and is rendered immobile (López-Arredondo *et al.*, 2014). This leads to excessive application of fertilizers which causes eutrophication of water bodies (Smith & Schindler, 2009).

Limiting the use of fertilizers in agriculture is necessary because it alone cannot maintain yields and we must reduce our agricultural footprint on earth (Foley *et al.*, 2011; Tilman *et al.*, 2002). The sustainability of the practice is also a major issue because the reserves of rock phosphate will be depleted by the next 50 years (Gilbert, 2009). We need to screen varieties for root hairs that are more effective in capturing nutrients in low input systems to lower the environmental impact of agriculture (Gilroy & Jones, 2000).

Plants with root hairs have a competitive edge in environments with low phosphorus as demonstrated by improved phosphorus uptake and plant biomass compared to competitors without root hairs (Bates & Lynch, 2001). Multiple studies have shown that higher root hair length is correlated with enhanced phosphorus uptake by plants (Haling *et al.*, 2013; Vandamme *et al.*, 2013; Brown *et al.*, 2012; Leitner, *et al.*, 2010; Zygalakis *et al.*, 2011). The best evidence for root hairs' correlation with enhanced phosphorus uptake is shown by studies that compare wild type plants with their root hairless mutant. In maize, rht3 mutants, that don't possess any root hairs, suffer from reduced yields in field trials (Hochholdinger *et al.*, 2008).

Low availability of phosphorus has been shown to increase the length and density of root hairs in Arabidopsis (López-Bucio *et al.*, 2003). Strict transcriptional control is in charge of root hairs' plasticity (Vijayakumar *et al.*, 2016). In wheat and barley genotypes, it was found that the genotypes with longer root hairs had higher yield potential in both low and high phosphorus environments (Gahoonia & Nielson, 2004). They were found to be crucial for providing tolerance to the plants in phosphorus deficiency and drought stress, with root hairless genotypes undergoing severe growth retardation (Brown *et al.*, 2012).

Studies show that a possibility exists that root hairs with higher length and density could be releasing phosphate solubilizing acids. This can mean that root hairs work together with the root system architecture for efficient phosphate capture (Lynch, 2019; Yan *et al.*, 2004).

An ideotype has been proposed based on the lifetime of root hairs for greater uptake of phosphorus. The location of root hair zone changes continuously as the root tip grows. This makes the life and functionality of individual root hairs quite short. By increasing the lifetime of root hairs, their acquisition of phosphorus can be increased (Brown *et al.*, 2013).

1.9.Phenotyping of Root Hairs

In order to apply breeding strategies and produce desirable cultivars, it is important to associate genetic data with phenotypic variations, which needs to be accurate and reproducible. Laboratory-based root phenotyping strategies are particularly helpful in finding variations within traits of root systems (Paez-Garcia *et al.*, 2015). Since root hairs are important for nutrient uptake and are used as a model of cell development, measuring and phenotyping their traits is helpful in breeding programs (Guichard *et al.*, 2019).

Root hairs emerge and are visible soon after germination in seedlings. This means that screening and analysis can be performed in a short time for a large number of seedlings grown in petri plates (Grierson *et al.*, 2014). Another way of screening seedlings for root hairs is the roll-ups method in which sterilized seeds can be placed in germination papers and rolled into a 'cigar roll' conformation. These rolls can be then placed in nutrient solution for a week or more (Strock *et al.*, 2019; Miguel *et al.*, 2013).

The roll-up method has been used to grow wheat seedlings for high throughput phenotyping of root hair traits using a portable microscope. Traits like root hair length and density can easily be measured using imaging with microscopes and using various software for measurement (Maqbool *et al.*, 2022).

1.10. Root Hair Transcriptomics

Any -omic analysis of a multicellular organ like root will be the average representation of different types of cells found in that organ. In this type of analysis cell-specific transcripts and responses cannot be uncovered (Qiao & Libault, 2013). Specific cells can be successfully isolated

using laser capture microdissection, but it is labor intensive and has lower yields (Takehisa *et al.*, 2012). This has led to the development of methods in which biology can be understood at the level of a single cell by using root hairs (Libault *et al.*, 2010a), even though isolating root hairs is challenging because of the accessibility of roots and the fragility of hairs.

One of these methods is to freeze the root system in liquid nitrogen and gently brushing the roots to fracture the root hairs (Ramos & Bisseling, 2003). This method is great for obtaining pure samples but the downside is the lower yield. The other method is stirring the roots in liquid nitrogen and then filtering it to obtain root hairs. (Qiao & Libault, 2013). This method yields more sample and is less labor intensive.

Root hairs specific transcriptome has been obtained and analyzed in various species. In soyabean, the root hair transcriptome has been compared to that of the root by Affymetrix arrays and Illumina sequencing. Expression of 48,281 predicted genes was quantified by sequencing and 11,807 by Affymetrix arrays. Illumina sequencing was clearly more thorough and allowed distinction between less abundant and absent transcripts (Libault *et al.*, 2010b).

Similar profiling was performed in Maize by analyzing the genetic composition of root hairs via RNA sequencing. It was found that 831 genes were exclusively expressed, and 5585 genes were preferentially expressed in root hairs (Hey *et al.*, 2017). Recently the root hair specific transcriptome was compared to the transcriptome of stripped roots in *Cicer arietinum* to study the plant's response to low phosphorus. The expression of genes involved in hair initiation and cell differentiation like *RSL* and *ROP guanine nucleotide exchange factor* (*ROPGEF*) was found in stripped roots and those involved in tip growth and cell wall formation like *EXPA2*, *GRP2*, and *XTH2* was found in root hairs (Kohli *et al.*, 2022).

1.11. Objective

The research objective of this study is to characterize the tissue-specific transcriptome of wheat root hairs and compare it to that of stripped roots.

CHAPTER 2
MATERIALS AND METHODS

2.1. Germplasm

In this study, wheat cv. Zincol-16 (Zincol)was used. Zincol was released in Pakistan in 2016 and it contains 20% more zinc than other high yielding varieties. Zincol was developed by the CIMMYT breeding program which aimed at producing elite cultivars by crossing high yield varieties with those containing high proportions of micronutrients (Singh et al., 2017).

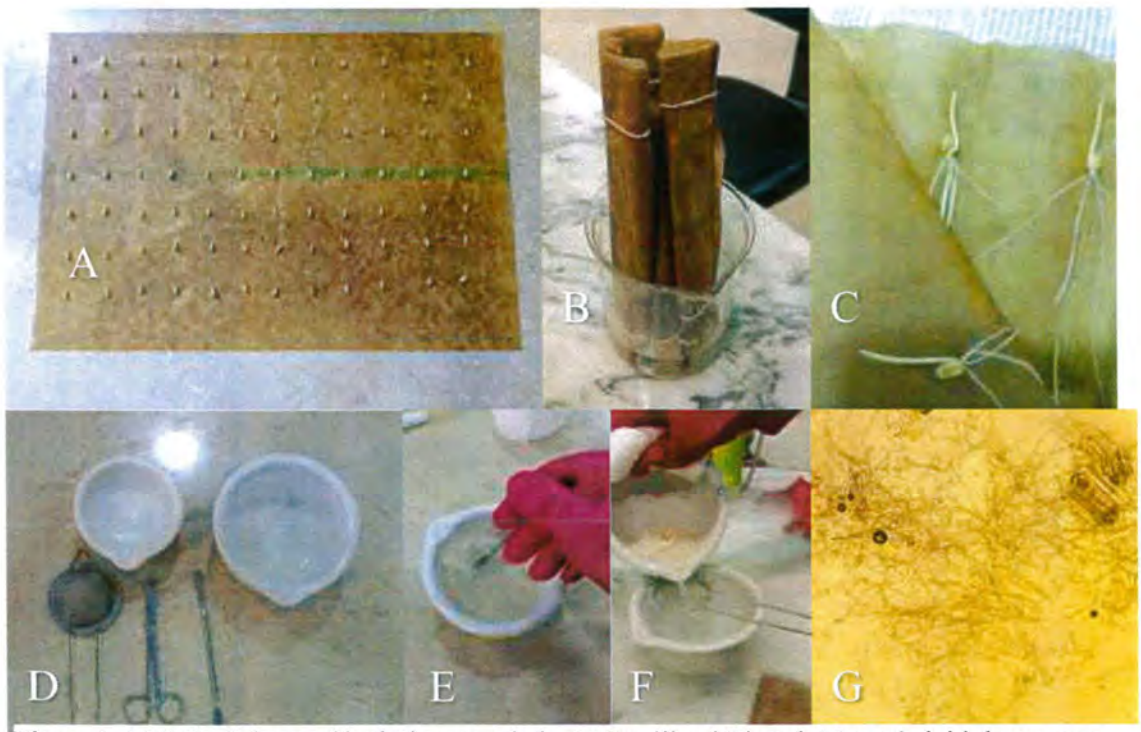

Figure 2.1 Methodology of isolating root hair. A: Sterilized Zincol-16 seeds laid down on a germination paper. B: Germination paper rolled into cigar rolls. C: Seedlings 4 days after sowing. D: Equipment used for isolating root hairs. E: Roots fragments being stirred in liquid nitrogen. F: Root hairs are filtered with a metal strainer into another mortar where the tissue would be crushed for RNA extraction. G: Isolated root hairs observed under microscope at 40x

2.2. Isolation of root hairs

Plants were sown using the cigar roll method. Seeds of Zincol were surface sterilized by soaking them in 5% sodium hypochlorite solution, followed by washing 2-3 times with distilled water. These seeds were then placed on wet Anchor germination paper, evenly spaced in rows. After laying another germination paper on top, these papers were rolled up carefully. These rolls were placed in beakers containing two types of treatments: high phosphate (0.25 mM KH_2PO_4, 0.10 mM KCl, 0.5 mM $CaSO_4$) and low phosphate (0.05 mM KH_2PO_4, 0.345 mM KCl, 0.5 mM $CaSO_4$). After 4 days of growth, the root systems of seedlings were harvested in a mortar containing liquid nitrogen. The liquid nitrogen was gently stirred with a metal rod to generate a flow that fractured the root hairs off the roots. The liquid nitrogen slurry containing the root hairs was passed through a metal strainer to another mortar to filter out any root fragments. Root hairs from 400-500 seedlings were collected and bulked into a single sample. Three biological replicates were used for each treatment. The roots stripped of hairs were also collected.

2.3. RNA extraction

Using pestle and mortar, the root hair and stripped root samples frozen in liquid nitrogen were grinded into a fine powder. TRIQuick Reagent was added to this powder and mixed with the sample. The mixture was incubated at room temperature for 15 minutes. The samples were transferred to Eppendorf tubes and centrifuged at 12000g for 15 minutes at 4 °C. The supernatant was pipetted into another Eppendorf tube and 200 µl chloroform was added to it and shaken rapidly. After leaving the samples at -20°C, they were centrifuged again at 12000g for 15 minutes. The upper aqueous phase was collected into another Eppendorf tube and isopropanol was added into it before incubating at -20 °C for 45 minutes. The samples were centrifuged again at the same settings and the supernatant was discarded. The RNA pellet was washed with 75% ethanol twice and air dried. It was resuspended in Nanopure water. Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) was used to assess the yield and purity of RNA samples. The samples were stored at -80 °C.

2.4. cDNA synthesis

ABClonal ABScript III RT Master Mix was used to synthesize cDNA of the RNA samples. The reaction mixture of 10 µl was comprised of 2 µL 5X ABScript III RT Mix, 0.5 µL 20X gDNA

remover mix, and 6.5µL nuclease-free water and 1 µl RNA. The PCR conditions were as follows: 37 °C for 2 minutes, 55 °C for 15 minutes, 85 °C for 5 minutes, and 4 °C till the end. cDNA was quantified using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The samples were stored at -20 °C.

2.5. Primer designing

Complete coding sequences of gene TraesCS1A02G313600 and its homeologues from B and D genomes were retrieved from IWGSC RefSeqv1.0. qRT-PCR primers were designed manually from these coding sequences. The sequences of these primers are given in Table 2.1.

Table 0.1 Primers used in the study.

Primers	Sequences	Product size
TraesCS1A02G313600-F	CCATGGTGAGAAAGAGGTG	163
TraesCS1A02G313600-R	GCGACATAGATGAACAATCC	
TraesCS1B02G325400-F	TCGCTCTGTCTTCTTCCTC	128
TraesCS1B02G325400-R	CTCAAGATCTAGTTCCTCGT	
TraesCS1D02G314100-F	GCCGAACAAGAAGTGCCTT	169
TraesCS1D02G314100-R	TCATCGTCGCCATCATCAC	
TaActin-F	CCGTTCTGTCCTTGTATGCC	140
TaActin-R	GCGTGTATCCCTCGTAGATTG	

2.6. qRT-PCR

Bio-Rad CFX384 Real-Time Touch Thermal Cycler was used for qRT-PCR to assess the relative gene expression of TraesCS1A02G313600 and its homeologues. Genious 2x SYBR green rapid qPCR mix (ABclonal) was used in the process with the primer for TaActin as control. The expression levels of the gene were measured by the Livak technique (Bio-Rad) in a CFX384 Real-Time detection system (Livak & Schmittgen, 2001).

Reaction mixture of 10 µL made for each sample for qPCR reaction contained the following components: 2X Universal SYBR Green Fast qPCR, 5 µL of master mix, 1 µL cDNA, 0.2 µL of forward primer, 0.2 µL of reverse primer and 3.6 µL of nuclease-free water. The reaction conditions were as follows: 1 cycle at 95°C for 3 minutes, 40 cycles at 95°C and 60°C for 5 and

30 seconds respectively, and then a melt curve of 58°C to 95°C for 5 seconds each using 0.5 °C increments. A standard curve was constructed after the validation of amplification and melting curves.

2.7. RNA sequencing

Root hair and stripped root samples were obtained as described in section 2.1. In addition to these, whole root and leaf samples were sent to Beijing Genomics Institute (BGI) for Illumina sequencing 1.9. The 250-bp paired-end sequencing libraries were constructed, and BGISEQ-500 platform was used for sequencing using standard protocols.

2.8. RNAseq analysis

RNAseq files were imported to the Galaxy public platform and the public server usegalaxy.eu was used to assess the data for quality, preprocessing, alignment to the genome and for generating raw counts (Afgan et al. 2016).

FastQC was used to assess the raw read files for quality and to generate quality reports (Version 0.12.1; Andrew, 2010). Trimmomatic (Version 0.38; Bolger et al, 2014) was used for quality trimming and cutting the adapters and other sequences specific to Illumina. The trimmed sequences were again assessed for their quality using FastQC. The trimmed reads were aligned to wheat's reference genome obtained from Ensembl (http://ensemblgenomes.org/) using HISAT2, a fast and sensitive alignment program (Version 2.2.1; Kim et al, 2015). The BAM files obtained from HISAT2 were used to count aligned reads using featureCounts (Version 2.0.3; Liao et al., 2013). For counting aligned reads, annotation file for IWGSC Refseq v2.1 was used.

The differential expression analysis and normalization of counts was performed using NOIseq with Pairwise Differential Expression Analysis (Without Replicates) (Version 4.3; Tarazona et al., 2015) in the OmicsBox desktop platform (Version 3.0.30; Bioinformatics & Valencia, 2019). Lowly expression genes were removed using counts per million (CPM) > 0.5. Using NOIseq, counts were normalized using the TMM method. Plots were constructed within using OmniBox.

2.9. Gene structure and phylogenetic analysis

Gene Structure Display Server (GSDS 2.0 http://gsds.gao-lab.org/) was used to construct the gene structure of TraesCS1A02G313600 and its homeologues.

The coding sequences of TraesCS1A02G313600 and its homeologues was taken from IWGSC RefSeqv1.0 blast database (https://urgi.versailles.inra.fr/blast_iwgsc/blastresult.php). The orthologues of this gene in *Hordeum vulgare*, *Oryza sativa indica*, *Oryza sativa japonica*, *Aegilops tauschii*, *Triticum urartu*, *Oryza glumipatula*, *Oryza barthii*, *Oryza nivara*, *Oryza punctata*, *Oryza glaberrima*, *Oryza rufipogon*, *Setaria italica*, *Secale cereale*, *Triticum turgidum*, *Setaria viridis*, *Leersia perrieri* and *Lolium perenne* were taken from NCBI (www.ncbi.nlm.nih.gov/) and Ensembl (http://ensemblgenomes.org/).

The orthologues were aligned using ClustalW in MegaX and the phylogenetic tree was constructed using neighbor joining method. The phylogenetic tree was then illustrated in Interactive Tree of Life (iTOL) v5 (Letunic & Bork, 2021).

CHAPTER 3
RESULTS

3.1 Transcriptome sequencing of root hairs of Zincol

The RNAseq data of root hairs from Zincol was of of 320.7 million fragments with an average GC content of 52% and average sequence length of 150bp. The Phred quality score for the sequences was 37 which corresponds to 99.98% base call accuracy (Figure 3.2). The reads were mapped to reference genome obtained from Ensembl using HISAT2 (Kim et al., 2015) and featureCounts (Liao et al., 2014) was used to count genes. The assigned library sizes ranged from 28 million in the leaf tissue to 32.9 million in the stripped roots.

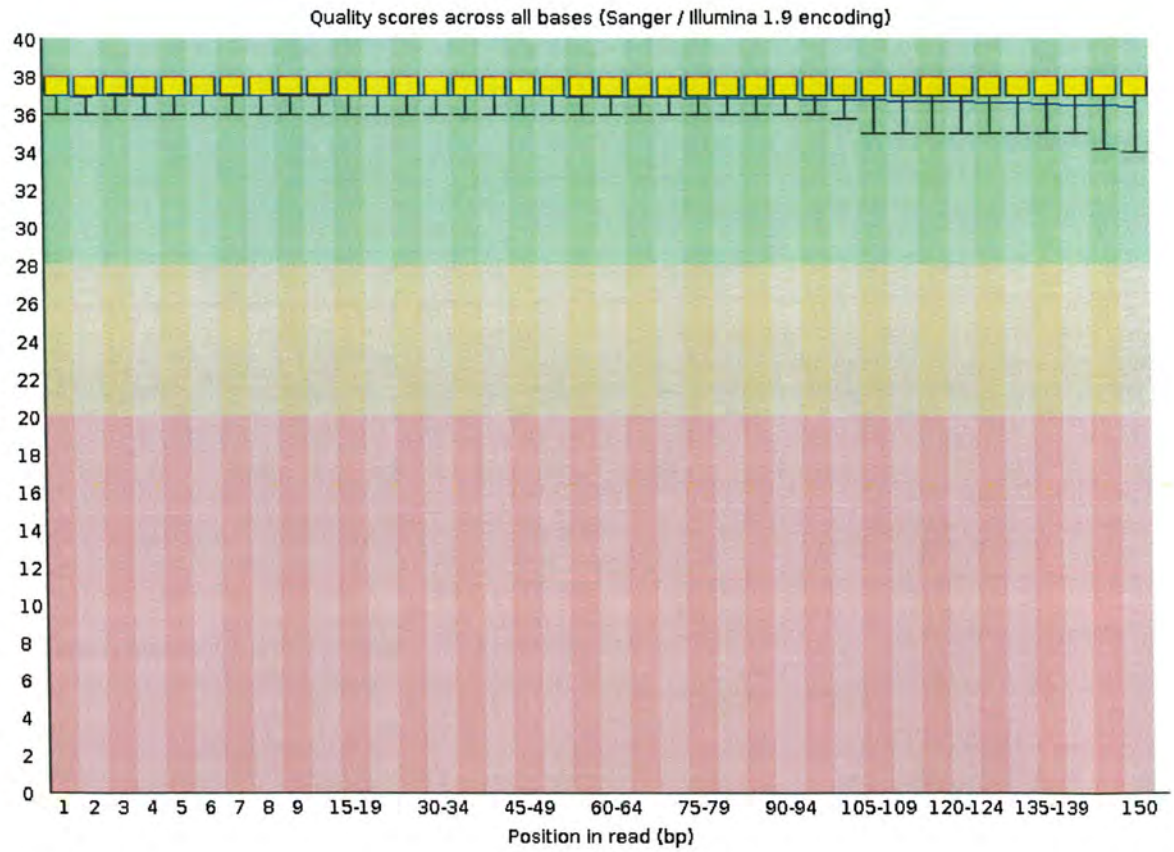

Figure 3.1. FastQC Results: Per base sequence quality for Root Hair sample from wheat cultivar Zincol.

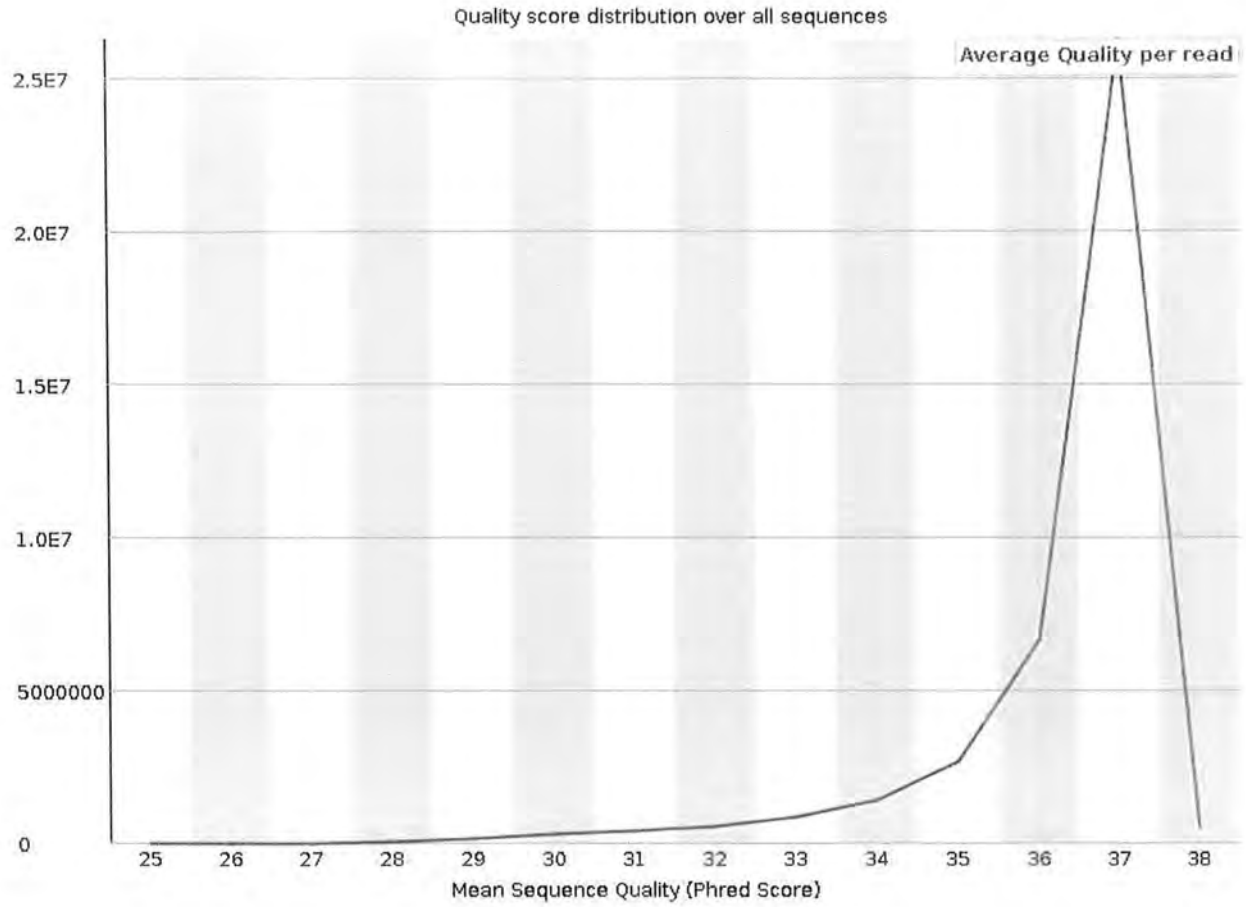

Figure 3.1. FastQC results: Per sequence quality scores – Phred Score for Root Hair sample from wheat cultivar Zincol.

Read counts were subjected to principal component analysis (PCA) using iDEP 1.1 and a plot was generated in a two-dimensional space where the axes are defined by the first and second principal components: PC1 and PC2. PC1 accounts for an impressive 90.4% of the total variance in the data, indicating that it captures the most significant source of variation which corresponds to the leaf and the root tissues (total root, root hair, stripped root). PC2 explaining 7.3% of the variation, which played a notable role in explaining the data's variability corresponding to the differences between root hairs, stripped roots and total roots (Figure 3.3).

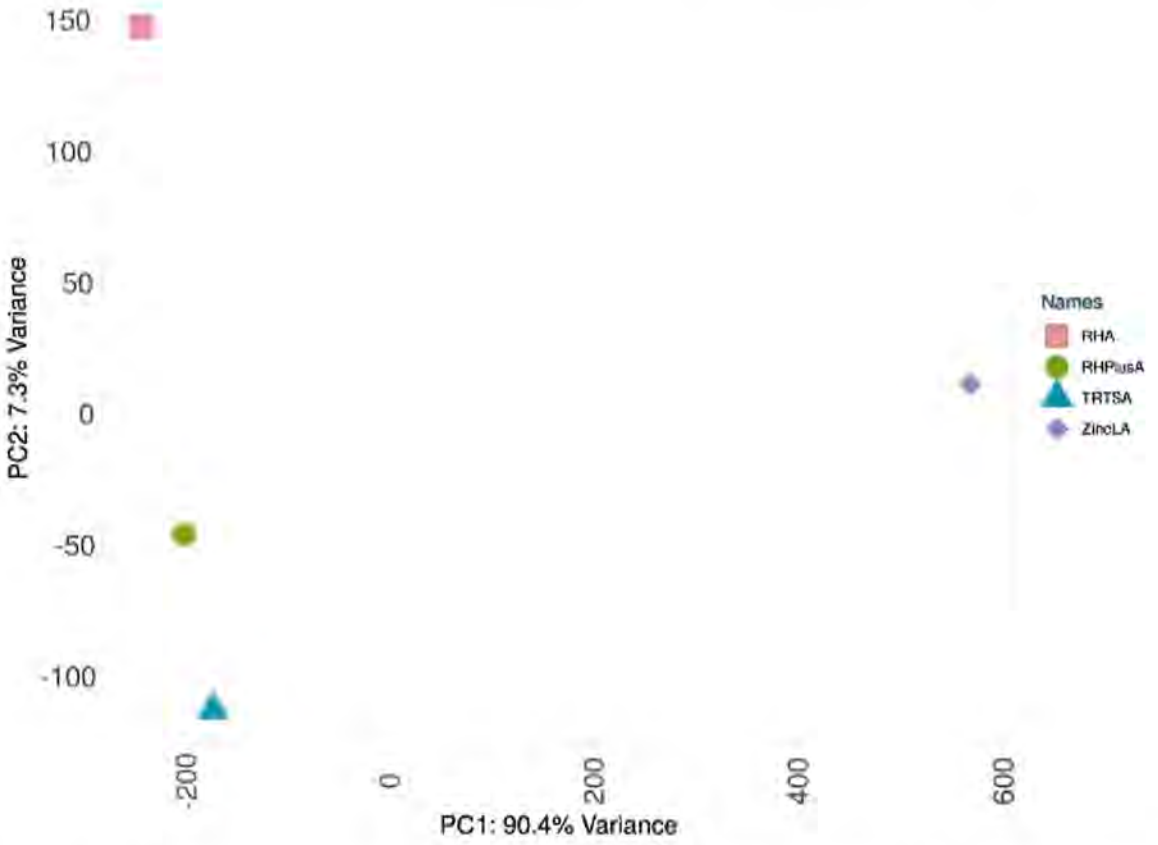

Figure 3.2. Principal Component Analysis (PCA) plot generated using iDEP 1.1; RHA: Root Hairs, RHPlusA: Stripped roots, TRTSA: Total Roots, ZincLA: Leaf tissue.

Differential expression analysis was performed using Pairwise Differential Expression Analysis (Without Replicates) in OmicsBox with stripped roots kept as reference and root hairs as the contrast. CPM filter was specified at 1.0 and TMM (Trimmed mean of M values) was used for normalization. Out of 107,891 features, 47,376 passed the filter. A multi-dimensional scaling or MDS plot was generated by OmicsBox which distanced root samples away from the leaf sample, demonstrating the disparity between them (Figure 3.5). The distances represented the log2 fold changes between the tissues. With probability threshold set at 0.9, 10,564 differentially expressed genes were found. 3,193 genes were upregulated and 7,371 were downregulated in root hairs with stripped roots as the reference condition.

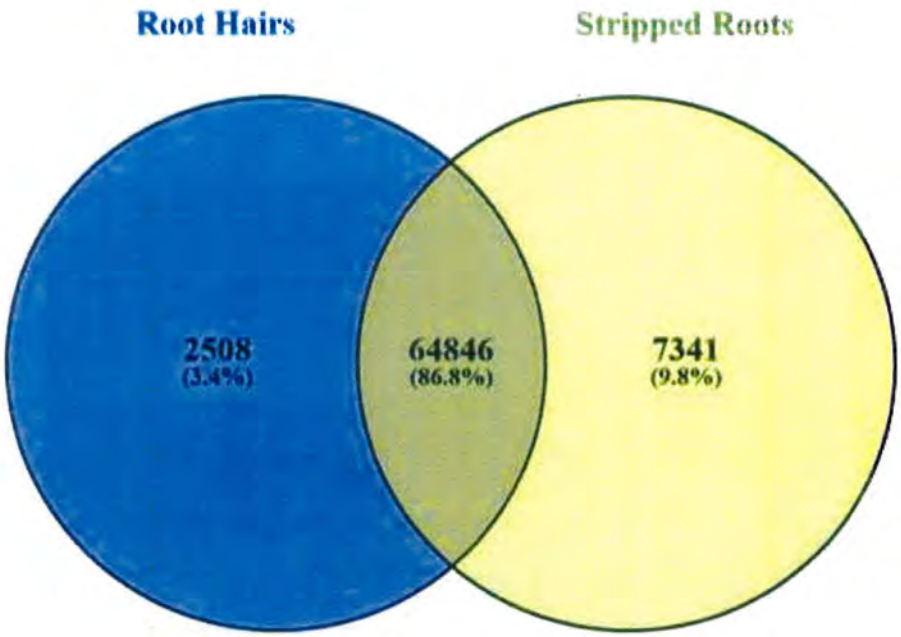

Figure 3.4. A Venn diagram showing the number of genes exclusively expressed in root hairs and stripped roots, and the genes commonly expressed in both.

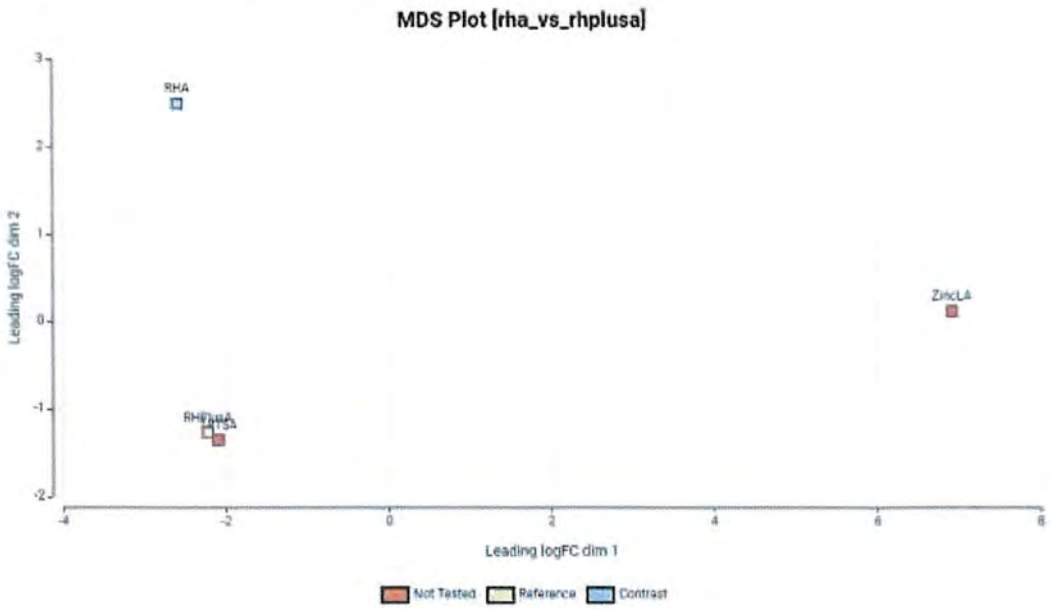

Figure 3.5. An MDS plot for the 4 tissues; the distances represent log2 fold changes between samples.

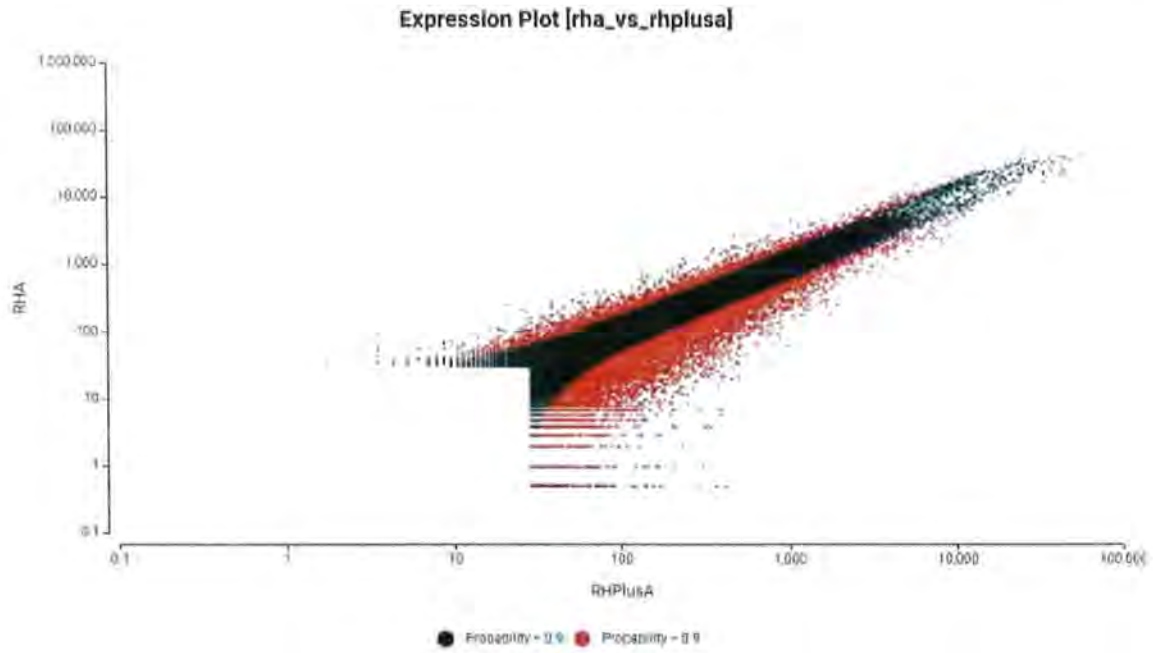

Figure 3.6. Expression Plot showing the average expression values for Root hairs and Stripped roots. Probability threshold was kept at 0.9. The differentially expressed features are highlighted in red.

Expression plot and MD plot were also generated. The former plot the average expression values for root hair tissue and stripped root tissue where the probability threshold was kept at 0.9. The common features are shown in black while the differentially expression features are highlighted in red (Figure 3.6). In the MD plot, M represents the log-fold change and D represents the absolute value of expression difference between root hairs and stripped roots in log scale (Figure 3.7).

Differential expression analysis was also performed with root hairs as the contrast condition and the total roots as the reference. This time 48,100 features passed the filter. 3,640 features were upregulated while 8,869 were downregulated in root hairs.

Heatmap for top 50 differentially expressed genes was constructed in OmicsBox. The heatmap demonstrated the expression differences between root tissues and leaf tissues (Figure 3.8).

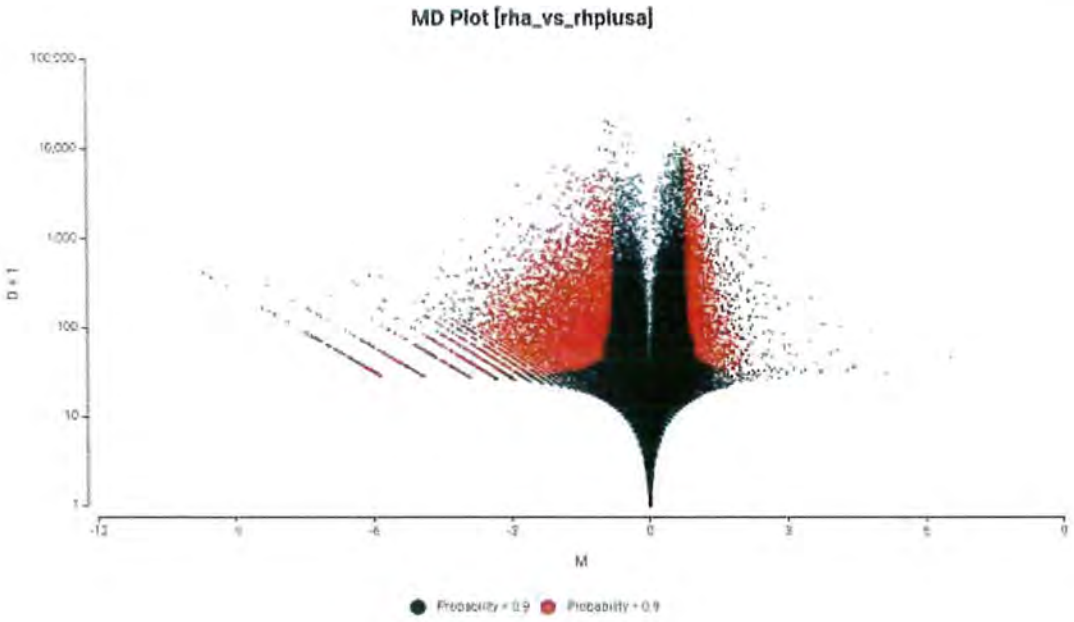

Figure 3.7. MD Plot where M= log fold change and D= difference in expression between Root hairs and Stripped roots.

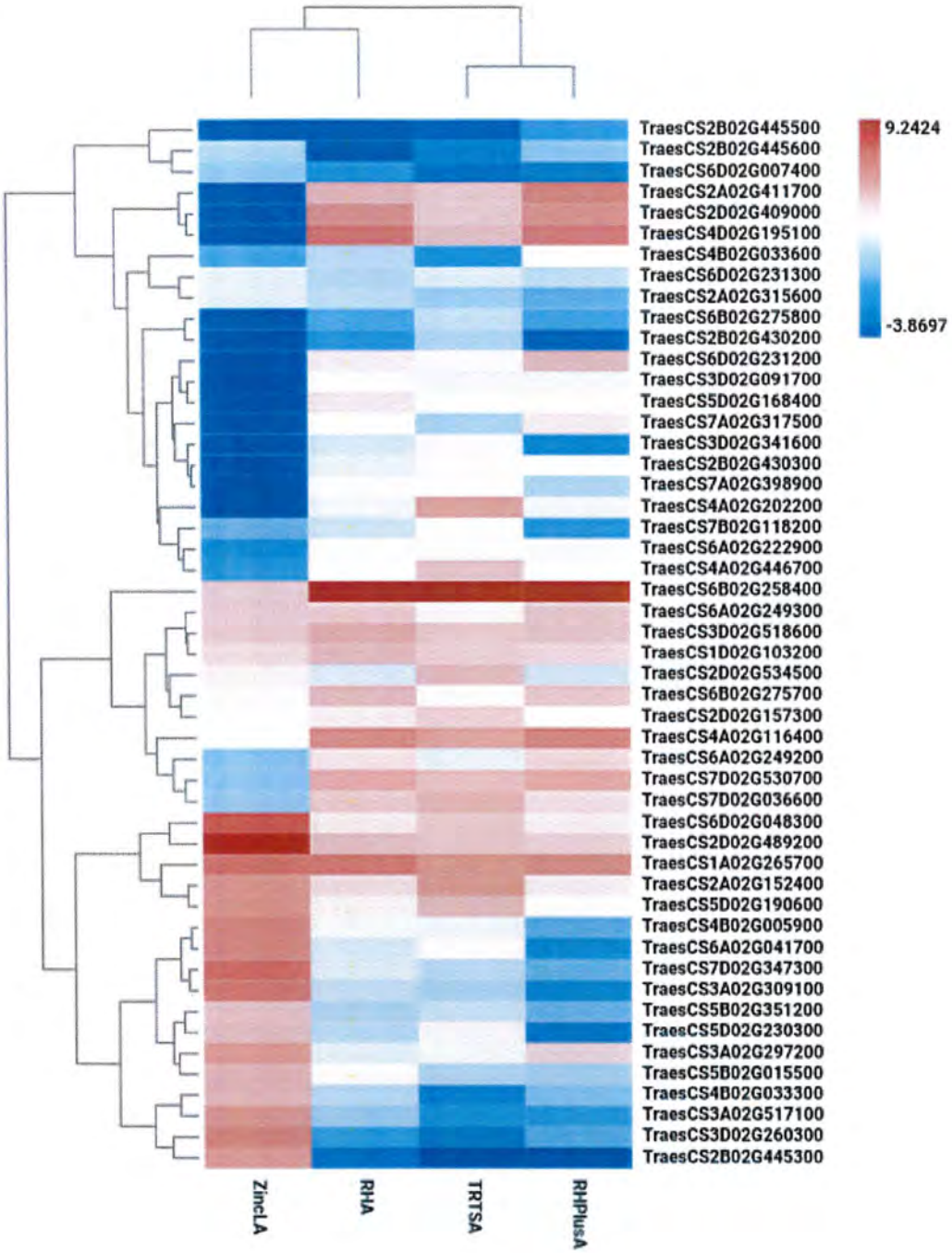

Figure 3.8. Heatmap of log2 Counts per million (log CPM) of the of top 50 differentially expressed genes across Root hairs, stripped roots, total roots and leaves of Zincol-16. The color scale is an indicator of the intensity of intrinsic expression (log CPM)

Expression levels of TraesCS1A02G313600, TraesCS1B02G325400 and TraesCS1D02G314100 were plotted in a bar graph using iDEP1.1. All three were expressed the lowest in leaf tissue. The B and D homeologues were expressed more in the root hairs compared to A homeologue (Figure 3.9).

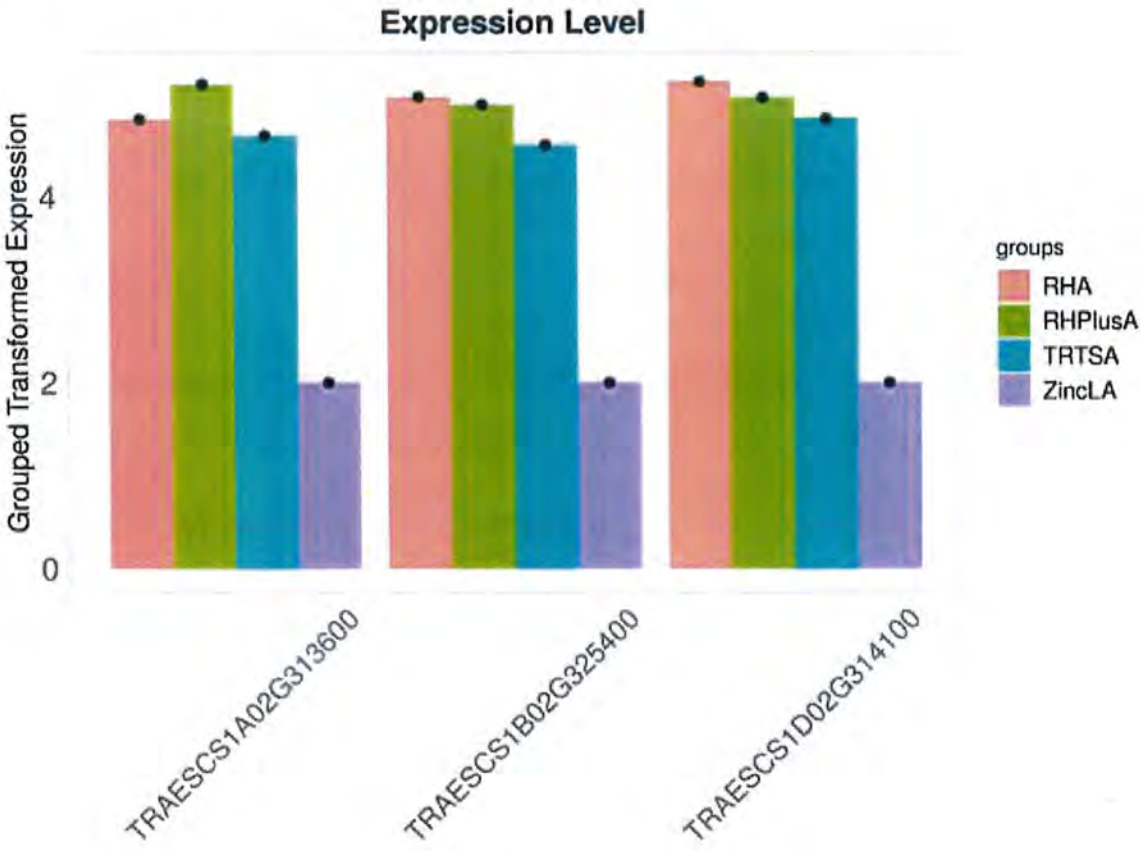

Figure 3.9. Expression levels of TraesCS1A02G313600, TraesCS1B02G325400 and TraesCS1D02G314100 in the four tissues.

3.1.1 Analysis in iDEP.96

iDEP.96 was used to generate scatter plots comparing the expression of all tissues with root hairs (Figure 3.10). The same platform was used to determine enrichment pathways. Using k-means method, 2000 most variable genes were clustered into 4 groups and the clusters were visualized into a tree (Figure 3.11). Gene Ontology or GO terms for biological processes were

determined using Parametric Gene Set Enrichment Analysis (PGSEA) with FDR set at 0.2 (Figure 3.11).

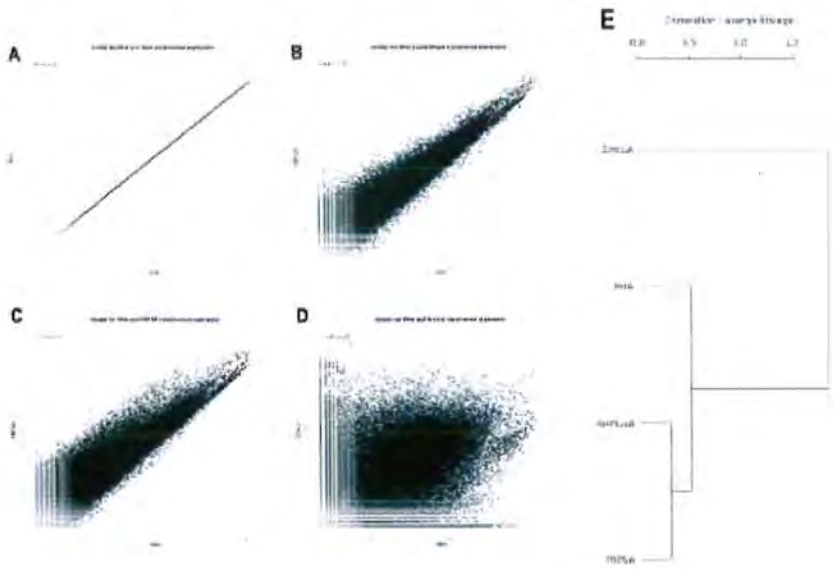

Figure 3.10 A-D: Scatter plots comparing the expression of root hairs with the rest of the tissue. E: Hierarchal clustering tree showing the correlation between four tissues.

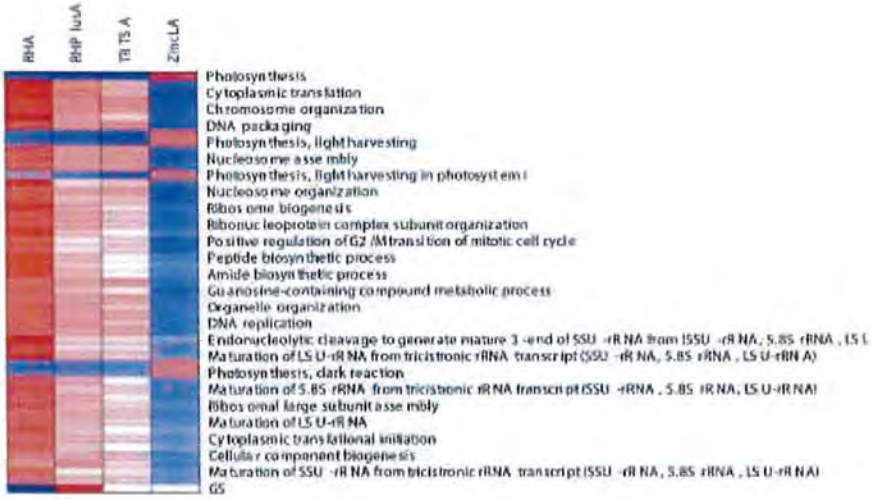

Figure 3.11 GO terms of biological processes using PGSEA method

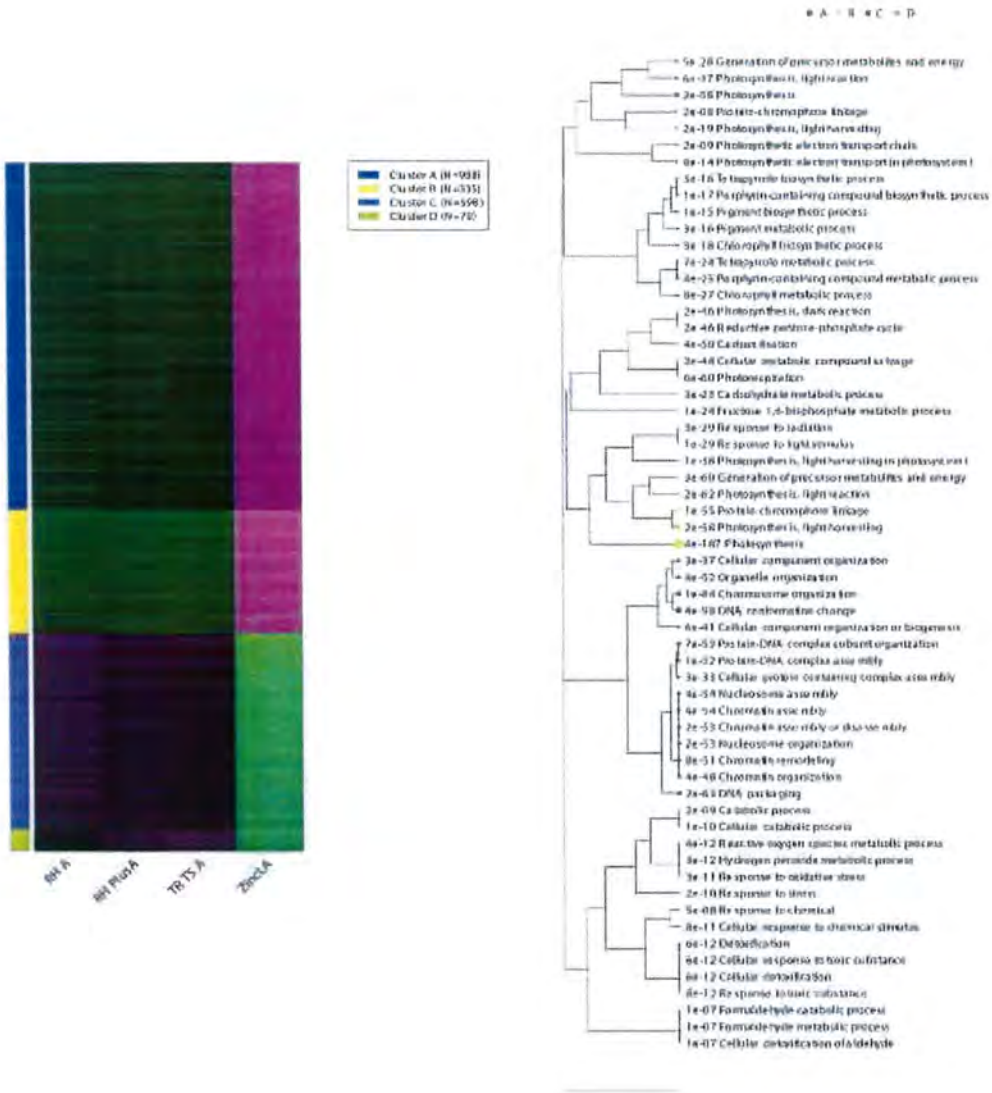

Figure 3.12. K-means clustering and Tree visualization of enrichment. Gene sets closer on the tree share more genes. Sizes of dot correspond to adjusted P values

Expression levels for genes linked with root hair length and density (Maqbool *et al.,* 2023) (Figure 3.13), expansins and xyloglucan endotransglucosylase/hydrolases (XTHs) (Figure 3.14) and genes associated with terms like 'root hair elongation', 'root hair initaition' etc. (Figure 3.15) were also plotted.

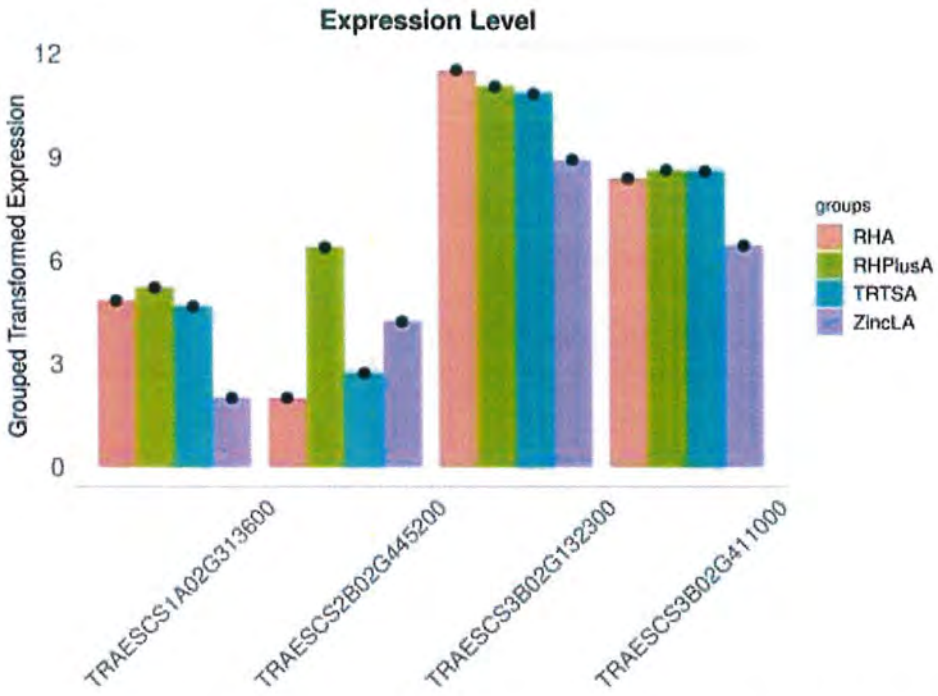

Figure 3.13 Expression levels of genes associated with root hair length and density

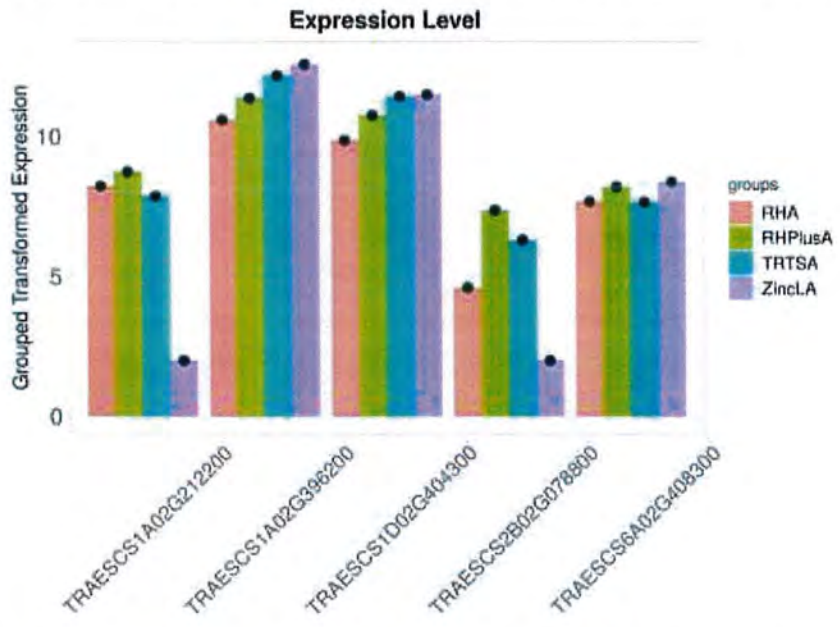

Figure 3.14 Expression levels for Expansins and xyloglucan endotransglucosylase/hydrolases (XTHs)

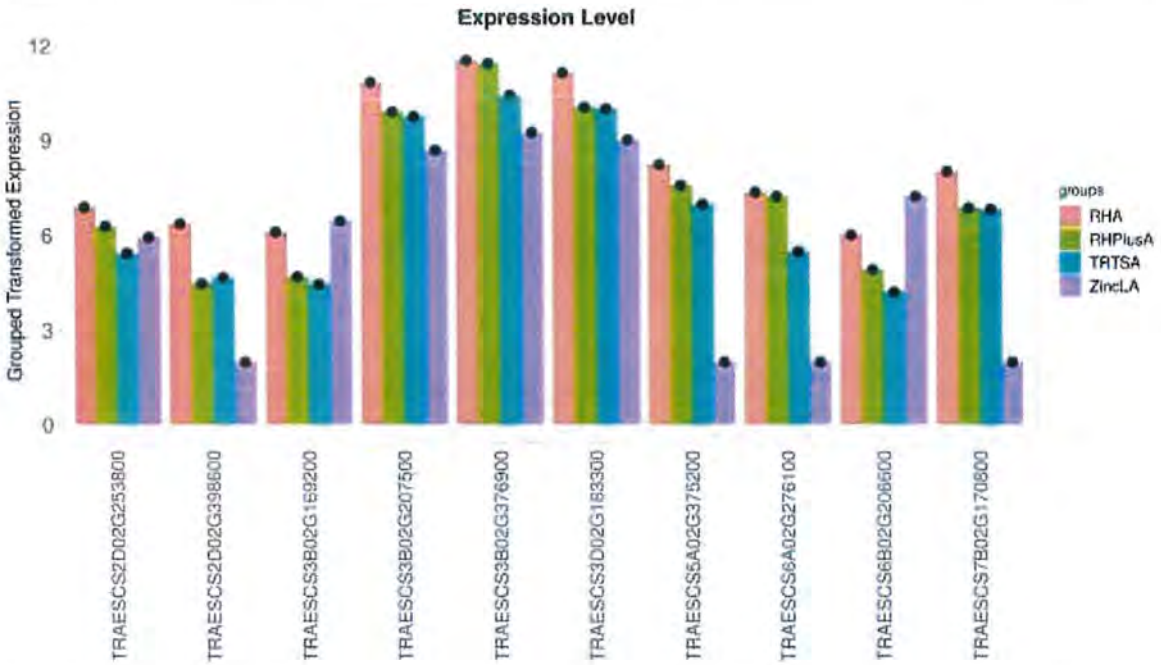

Figure 03.15 Expression levels of genes associated with terms like 'root hair elongation', 'root hair cell development', root hair cell differentiation' and 'root hair initiation'

3.2 Identification of gene structure and phylogenetic analysis

The gene structure of the three homeologues was constructed using Gene Structure Display Server (GSDS 2.0). TraesCS1A02G313600 and its homeologues are composed of one exon each (Figure 3.16). The total gene length of TraesCS1A02G313600 is 1278 bp, its B homeologue is 1313 bp and the D homeologue is 1333 bp long. These gene lengths include the length of upstream and downstream regions as well as exons.

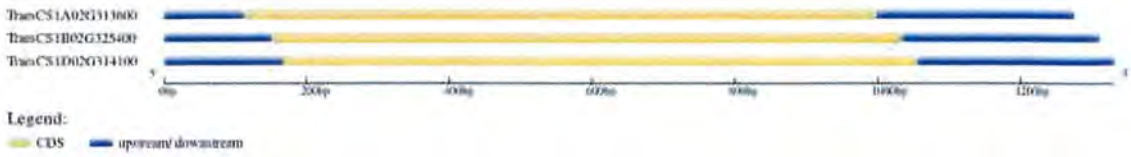

Figure 3.16 Gene structures of the three homoeologues designed in Gene Structure Display Server (GSDS 2.0)

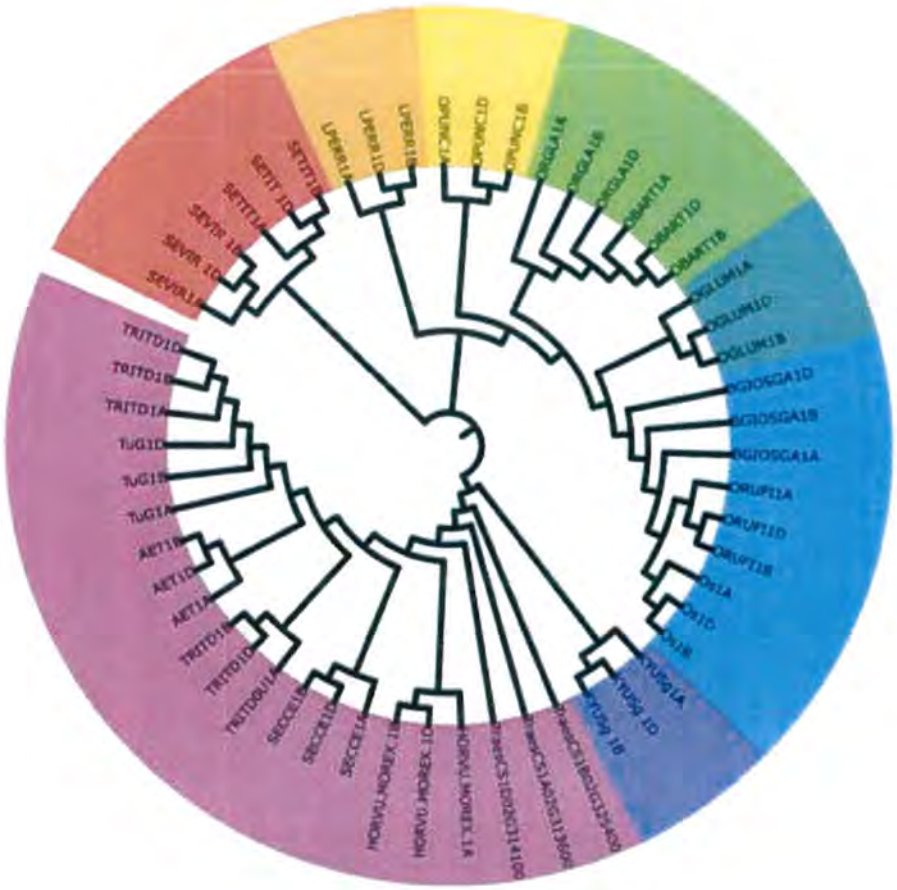

Figure 3.17. Phylogenetic reconstruction of the three homeologues and their orthologues. The phylogenetic tree was constructed using neighbor-joining method following ClustalW alignment using MegaX

Phylogenetic tree constructed through ClustalW alignment in MegaX and visualized in Interactive Tree Of Life (iTOL) v5 divided the orthologues of the three genes into three major clades: orthologues of orthologues of *T. aestivum*, *T. urartu*, *T. turgidum*, *Aegilops tauchii*, *Hordeum vulgare*, *Secale cereale*, and *Lolium perenne* into the first clade, orthologues of all the *Oryza sativa*, *O. glumipatula*, *O. barthii*, *O. punctata*, *O. glaberrima*, *O. rufipogon*, and *Leersia perrieri* into the second, and *Setaria italica* and *Setaria virida* into the third clade. The phylo-tree shows that TraesCS1A02G313600, TraesCS1B02G325400 and TraesCS1D02G314100 belong to the first clade, sharing evolutionary ancestory with Hordeum vulgare, Secale cereal and other

Triticum species. The D-homeologue is more closely related to the gene's orthologue in H. vulgare and S. cereale than the other two homeologues (Figure 3.17).

3.3 Expression analysis using qRT-PCR

qRT-PCR was used to analyze the expression of TraesCS1A02G313600 and its homeologues in root hairs and stripped roots of Zincol-16 under high and low phosphate conditions. In root hairs, under high phosphate conditions, the D homeologue was expressed the highest. Overall, the expression of B and D homeologues was the highest in root hairs and stripped roots under both high and low phosphate conditions. The B homeologue was expressed more under the low phosphate condition in both root hairs and stripped roots. The expression of A homeologue was much lower under both conditions in both tissue types compared to the B and D homeologues (Figure 3.18).

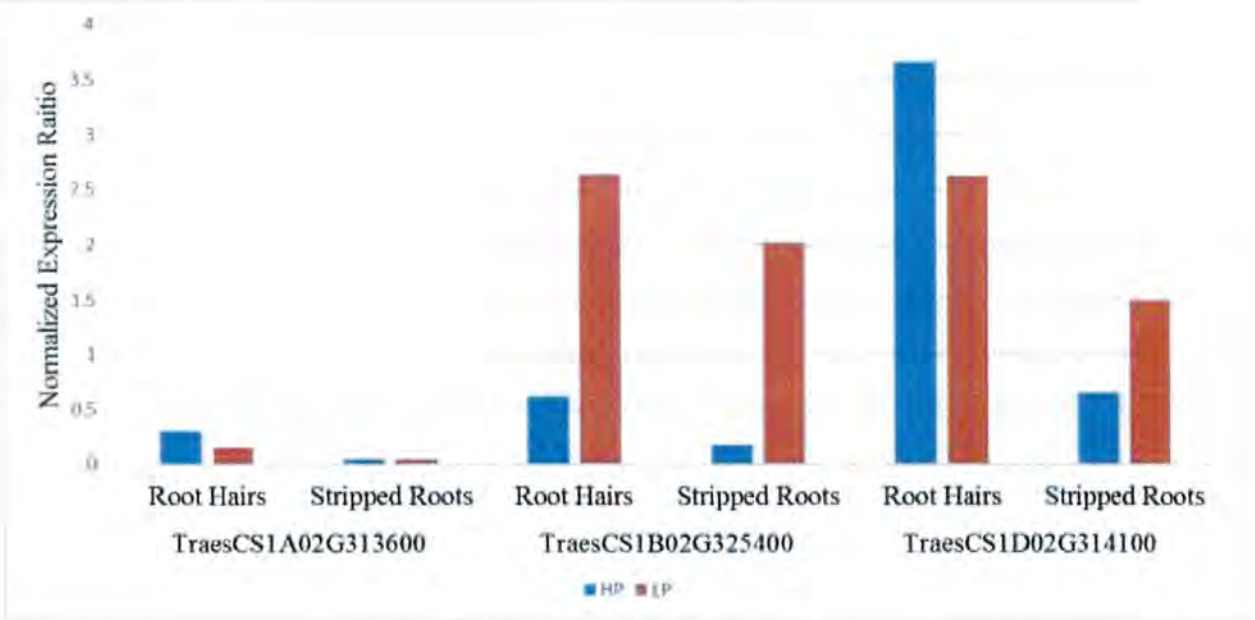

Figure 0.18. Relative expression of *TraesCS1A02G313600*, *TraesCS1B02G325400* and *TraesCS1D02G314100* under low and high phosphate in Zincol. Each sample includes three biological replicates.

CHAPTER 4
DISCUSSION

Root hairs are specialized protrusions of epidermal cells of roots that increase their surface area for increased absorption of water and minerals. Root hairs can serve as a remarkable model for studying cell biology, cell expansion, differentiation, and physiology (Grierson *et al.*, 2014).

Functional genomic studies like transcriptomics in plants are mostly focused on assessing the responses of entire plants, organs, or tissues. The results of these studies include signals from all the different kinds of cells, diluting the responses of singular, specialized cells like root hairs. There is a demand for methodologies that allow the utilization of the complete range of -omics based tools on individual cells instead of entire organs or tissues. Root hair cells are an appealing candidate because of their involvement in important tasks like water absorption, nutrient uptake and because they are not required for the viability of a plant (Libault *et al.*, 2010).

Various techniques have been employed to isolate root hairs of different species like soybean, maize, chickpea etc. Some of these techniques are labor intensive and some result in lower yields. For this transcriptomic study of root hairs, roots were stirred in liquid nitrogen and the slurry was filtered with a metal strainer to isolate root hairs. The root fragments stripped of hairs were also stored and sent for RNA sequencing along with root hairs, total roots and leaves.

This study used the Pakistani wheat cultivar Zincol, which was introduced in Pakistan in 2016 by CIMMYT. Zincol stands out due to its 20% higher zinc content compared to other high-yielding variants. This was achieved by crossing high-yield germplasm with those rich in essential micronutrients such as zinc, addressing the deficiency of these elements in the diets of individuals from South Asian regions (*Singh et al.*, 2017).

Through pairwise differential analysis in OmicsBox between root hairs and stripped roots, 3,193 genes were preferentially expressed in root hairs while 7,371 were downregulated. Expressed genes were compared in root hairs and stripped roots using a Venn diagram (Venny 2.1.0; Oliveros, 2007). It was found that 3.4% genes were exclusively expressed in root hairs while 9.3% were expressed in stripped roots. This finding closely resembles the results seen in other

species like 5% in Arabidopsis (Lan *et al.*, 2013), 3% in maize (Hey *et al.*, 2017) and 2.2% in chickpea (Kohli *et al.*, 2022).

TraesCS1A02G313600 is responsible for coding a protein with a C2H2-type domain. This gene has been linked to the elongation of root hairs under low phosphate levels. Proteins that contain C2H2-type domains are characterized by zinc finger motifs. These proteins significantly regulate the expression of genes associated with abiotic stress responses in plants (Han *et al.*, 2020). Additionally, they facilitate the growth and development of root hairs by overseeing the activity of specific target genes. Other than this gene, other genes associated with root hair length (TraesCS2B02G445200, TraesCS3B02G411000) and root hair density were (TraesCS3B02G132300) were also identified (Maqbool *et al.*, 2023) and compared for their expression levels in the four tissues. The expression of the gene associated with root hair density had the highest expression in all four tissues. Overall, the genes associated with root hair length were expressed the highest in stripped roots.

Expansins and xyloglucan endotransglucosylase/hydrolases (XTHs) are proteins associated with the growth of cell wall (Liu et al., 2007). The expression levels of TaExpA1, TaExpB5, TaXTH1, TaXTH2, TaXTH4 were compared using a bar plot. The expressions of TaExpA1 and Ta ExpB5 were the most interesting as they were least expressed in leaves and most expressed in stripped roots.

Gene ontology terms were determined for genes upregulated in root hair exclusive using Singular Enrichment Analysis (SEA) in agriGO (Tian *et al.*, 2017). 168 genes were assigned to the term 'negative regulation of biosynthetic process'. Among these genes TraesCS6B02G206600 was associated with the term 'root hair elongation'.

For the GO enrichment analysis, terms like 'protein metabolic process', 'reproductive structure development' and 'photosynthesis' were significantly enriched. From the genes found in the enriched terms, genes for terms like 'root hair elongation', 'root hair cell development', root hair cell differentiation' and 'root hair initiation' were identified and plotted for their expression levels. TraesCS3B02G207500, TraesCS3B02G376900, TraesCS3D02G183300, TraesCS5A02G375200, TraesCS6A02G276100 and TraesCS7B02G170800 followed a similar

pattern; root hairs having the highest expression, followed by stripped roots, total roots and then the leaves showing the lowest expression.

CONCLUSION

This RNAseq study has provided valuable insights into the transcriptome of root hairs and stripped roots of wheat cv. Zincol. The study explored the differential expression of different genes associated with root hairs. While this study has contributed substantially to our understanding of the root hair transcriptome, it is important to note that in conducting this study, biological replicates were not employed, which limited the options of differential expression and other downstream analysis. However, these limitations also serve as opportunities for future research.

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