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TRANSCRIPTOMIC ANALYSIS OF PHOSPHATE UTILIZATION *OsRHL1* TRANSGENIC LINES OF RICE

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Abstract

Understanding and optimising the response of plants to soil nutrient conditions is of central importance in enhancing food security. Root hairs are known to play important role for the uptake of nutrients and water from the rhizosphere and therefore worthy of study.

OsRHL1 is a novel basic helix-loop-helix transcription factor that regulates root hair development in rice. Previous studies from Professor Ping Wu's group (Ding et al., 2008)'s group show that *OsRHL1* may regulate genes involved in both root hair elongation and epidermal cell patterning in rice. These studies have shown the important role of *OsRHL1* in enhancing root hair growth and phosphate uptake in hydroponic growing systems. Further study for *OsRHL1* transgenic line growing in soil was therefore suggested.

The overarching aim of this project is to understand the function of the *OsRHL1* gene through transcriptomic approaches. Firstly, the gene expression pattern of *OsRHL1* was studied using transcriptomic data. The microarray data from *OsRHL1* over-expression transgenic rice line and its wild type control was then used to map out the components of the gene network that regulates root hair development in rice.

Results obtained from Gene Expression Omibus (GEO) and GeneVestigator using available transcriptomic data suggest that the expression of *OsRHL1* is mainly in the root and also present in floral pollen which was not reported previously (e.g. Ding et al. 2008). The gene was found to be involved in the drought and salt tolerance pathways, as well as hormone cytokinins pathway. Furthermore, results obtained from the microarray data analysis of *OsRHL1* over-expression and wild type KAS control indicate that *OsRHL1* interacts with a series of genes involved in cell wall preproduction, material transporting, and genes that regulate root hair tip growth. It also suggests that a key gene involved in phosphate uptake *OsSPX2* is among the components of

gene networks contributing to long root hairs in *OsRHL1* over-expression transgenic line.

The hydroponic growth experiments undertaken in this work suggests that root hair length does play a role in phosphate uptake. *OsRHL1* over-expression transgenic plant with long root hair was found to be associated with increased phosphate uptake and resulted in longer shoots and better root growth in low phosphate conditions. The total shoot phosphate content in the *OsRHL1* over-expression transgenic line is also significantly higher than the wild type control and its short-hair mutant *rhl1*. This trend was not detected in hydroponic solution with high levels of phosphate. This result suggests that the length of root hair does play an important role in root phosphate uptake in plant growth medium when the phosphate is less available. This role is less important when there is sufficient phosphate in the medium.

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Chapter 1: Introduction

1.1 Background

As the outgrowths of root epidermal cells, root hairs play an important role in water and nutrient uptake, plant anchorage, and microbe interaction by providng a large surface area. Root hairs are also an ideal model to study patterning mechanisms by investigating how epidermal cells adopt different cell fates to become either hair-bearing or non-hair bearing cells. Understanding the mechanism of root hair development in rice at the genetic and molecular level could potentially contribute to optimising rice yield and food security.

A number of genes involved in rice root hair development have been identified using genetic approaches, including Os*RH2* (Suzuki et al., 2003), *OsAPY* (Yuo et al., 2009), *OsCSLD1* (Kim et al., 2007), *OsPHR2* (Wu and Wang, 2008), ROOT HAIR DEFECTIVE-SIX LIKE (RSL) (Kim et al., 2016), ROOT HAIR SPECIFIC 10 (RHS10) (Ywang et al., 2016). However, compared to *Arabidopsis*, there is still a lack of understanding of the gene regulatory networks in this important staple crop.

In contrast, the gene regulatory networks underlying root hair growth in the model plant *Arabidopsis* have been well studied using genetic approaches, especially in terms of the patterning determination of root epidermis and growing processes including root hair initiation and elongation.

Although the root epidermal patterning in *Arabidopsis* is different from those in rice, the root hair initiation and elongation processes are expected to share similar mechanisms. The homologous genes which were found to play roles in root hair initiation and elongation process, could also serve the same purpose in rice. Understanding the mechanism of root hair development in rice will also help us to explore

the differences in root hair growth mechanisms between monocotyledon and dicotyledon plants.

1.2 *Arabidopsis* root hair growth gene regulatory network

1.2.1 *Arabidopsis* root hair patterning

There are three stages in root hair formation: patterning, initiation and elongation. Patterning determines which root epidermal cells develop into root hair bearing cells and it happens at the root meristem at a very early stage (Dolan et al., 1993, Schiefelbein, 2001 and Pattanaik et al., 2014). The regulatory network controlling the assignment of cell fate is characterized by the interaction between key proteins GLABRA3 (*GL3)*, ENHANCER OF GLABRA3 (*EGL3)*, and TRANSPARENT TESTA GLABRA (*TTG)* which form protein complexes by binding either WEREWOLF (*WER)* or CAPRICE (*CPC)* to determinate epidermal cell fate (Pattanaik et al., 2014).

Arabidopsis has a striped root hair pattern with epidermal cell files bearing root hairs (H cells) separated by non-hair bearing cell files (N cells) (Figure1.1 A). This pattern is position-dependent as H cells are always located over the crevice between two underlying cortical cells while N cells touch only one cortical cell (Berger et al., 1998a) (Figure1.1 B). Root hairs grow from root epidermal cells which in turn are derived from lateral and epidermal initial cells (LE initial cells) located in the root meristem zone in *Arabidopsis.* There are 16 LE initial cells in a ring around 8 cortical initials (Dolan et al., 1993). Each LE initial cell periclinally divides first to generate an outer and inner cell. The inner cell then undergoes an anticlinal cell division to give rise to an epidermal cell and a new initial cell (Dolan et al., 1993). With the continuation of the same pattern of cell division, a file of epidermal cells along the direction of root growth is produced by each LE initial cells. Occasional anticlinal cell division of epidermal initial cells will increase the number of cells in an epidermal ring, resulting in up to 22 cells contained in a mature epidermis ring (Berger et al., 1998b).

The two types of epidermal cells are trichoblast which later becomes H cells, and atrichoblast from which the N cells develop. Evidence suggests that the determination of epidermal cell fate occurs well before the appearance of the root hairs and the difference between trichoblast and atrichoblast can be distinguished in a number of features (Grierson and Schiefelbein, 2002). In fact, the expression of *GL2::GFP*, expressed in N cells only, can be traced back to the embryogenesis as early as at the early heart stage (Lin and Schiefelbein, 2001).

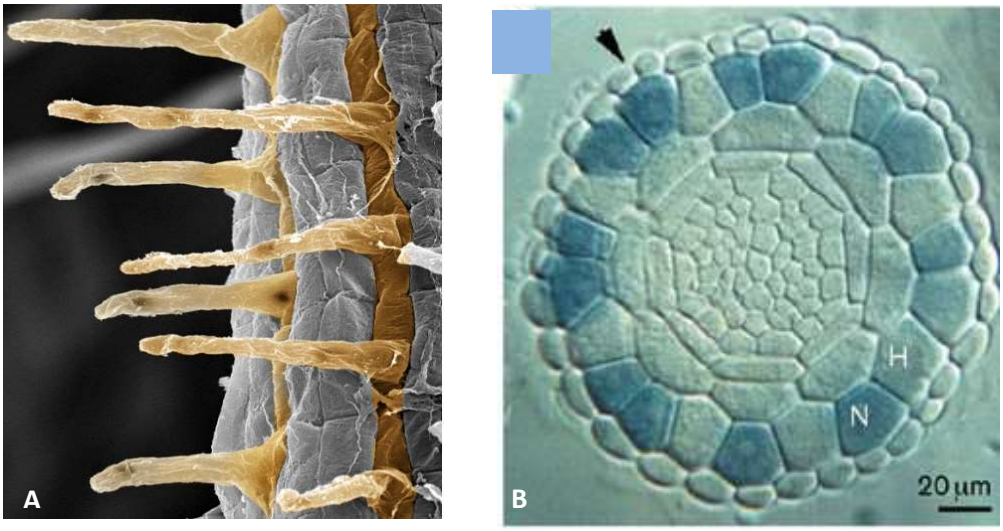


Figure 1.1: *Arabidopsis* root epidermal pattering. A: Striped root hair pattern in *Arabidopsis* (Benítez et al., 2010). B: Transverse section of *Arabidopsis* root showing the preferential expression of the *GL2::GUS* reporter fusion (green) in N cells only (Berger et al., 1998a).

The position-dependent cell fate determination continues post-embryonically to ensure the correct cell specification in the *Arabidopsis* root epidermis. Laser ablation of specific epidermal cells results in neighbour cells moving in to occupy the position; if this results in a change in position relative to the cortical cells, then the cell adapts its fate according to its new position rather than its old (Berger et al., 1998a). However, the correct cell fate determination does not need to be maintained by the continuous communication between epidermal cells in adjacent and underlying cortical cells (Berger et al., 1998a).

This determination is independent from continuous communication between adjacent cells.

1.2.2 *Arabidopsis* root hair initiation

Root hair growth is initiated from H cells. Before the appearance of root hair, Plant small monomeric G-proteins (*RAC/ROPs)*, *RHO-like GTPases* belonging to a family of related monomeric GTP-binding proteins, accumulate at the initiation site (Molendijk et al., 2001). These *GTPases* are controlled by hormones auxin and abscisic acid to signal hormone-regulated responses (Molendijk et al., 2001). *RAC/ROPs* interact with multiple transmembrane spanning *NADPH* oxidase to activate reactive oxygen species (*ROS*). *ROS* are found to accumulate at the tip of the emerging and elongating root hairs. Over-expression of *RAC/ROPs* results in the ectopic accumulation of *NADPH* oxidase-derived *ROS* and defective root hair phenotype (Duan et al., 2010).

The accumulation of Ca^{+} in the cell triggers the changes of pH in the cell wall which triggers the production of *ROS*. This change of pH also activates expansion proteins to loose the cell wall (Gabriele et al., 2009). The maintenance of the high concentration of Ca^{+} and *ROS* were found to be essential in the tip growth where new cell wall material is synthesised and secreted to the tip until growth ceases. The direction of root hair growth is controlled by a number of factors including calcium gradient, *RAC/ROPs* proteins and Microtubules (Grierson and Schiefelbein, 2002).

1.2.3 *Arabidopsis* root hair elongation

Tip growth involves exocytosis of plasma membrane vesicles and cell wall compounds. As the bulge expands, ER and actin cytoskeleton accumulates, maintaining the direction of the growth. Auxin also regulates the localization and accumulation of *ROP* GTPase, which in

turn regulates the organization of the actin and microtubule filaments (Ishida et al., 2008).

Modification of cell wall genes is an important part of root hair growth. The genes related to this process include Xylogulcan endotransglycosylases XETs, cellulose synthase-like protein, *AtCSLD3* in *Arabidopsis*, *OsCSLD1* in rice, UDP-D-galactose-4epiderase (*UGE4*), and a chimeric leucine-rice repeat extension-like protein (*LRX1*) (Ishida et al., 2008).

1.3 The role of plant hormones in *Arabidopsis* root hair formation

The plant hormones auxin and ethylene are positive regulators of root hair initiation. The inhibition of ethylene and auxin synthesis results in the loss of root hair growth (Masucci and Schiefelbein, 1994; Tanimoto et al., 1995, Hu et al., 2017).

The role of auxin in root hair cells elongation is indicated by the fact that the root hair length increases with auxin concentration (Pitts et al., 1998). However, active auxin influx has not been found in root hair cells despite the relation between them. This suggests that the concentration of *AUX1* in non hair cells is important for root hair elongation (Jones et al., 2009). The redistribution of auxin along the root also affects the root hair positioning. The disruption of the auxin gradient using *CTR1*, which is the negative translator of auxin biosynthesis, results in repositioning of the root hairs (Ikeda et al., 2009).

Both hormones are involved in the determination of root epidermal cell fate. Plants under treatment with ethylene or ethylene precursor *ACC* grow root hairs in the non-hair cell position (Tanimoto et al., 1995). Mutants in ethylene and auxin have reduced the difference in cytology between two epidermal cells type (Masucci et al., 1996). Plant steroid hormones BRs are also found to be required for the expression of *WER*

and *GL2*. Loss of BRs signalling results in the loss of root hairs in the H cell position (Kuppusamy et al., 2009). The relationship between hormones signalling pathway and *GL2* pathway is not clear. There is evidence suggesting that *GL2/TTG* pathway is not regulated by auxin and ethylene (Masucci et al., 1996). However, experiments performed by Niu et al. (2011) indicates that auxin signalling pathway is important for the elevated CO2 which regulates the expression of key genes in the *GL2* pathway.

1.4 Rice root hair development gene regulatory network

As a monocotyledon species, rice root hair patterning is very different from that of *Arabidopsis,* a model dicotyledon plant. Instead of being a striped type, every cell file in rice roots contains root hairs with a H cell (the shorter cell) adjacent to an N cell (the longer cell) (Rebouillat et al., 2009). The size difference between H cells and N cells implies that a late, asymmetrical transverse cell division gives rise to two daughter cells which adapt to different cell fates in rice. Despite the difference in the mechanism of cell fate determination, the genes encoding the cell wall-loosening protein EXPANSIN A (*EXPA*) with root hair-specific cis-elements (*RHE*) motif have found to have hair cell specific expression and have a function related to root hair elongation in rice (Yu et al., 2011), *Arabidopsis* (Kim et al., 2006) and grass (Won et al., 2010), suggesting the conservation of the root hair specific genes between monocotyledon and dicotyledon plants.

Compared to the root hair development network in *Arabidopsis*, only a few genes have been found to be involved in rice root hair development to date. One example is *OsAPY,* reported by Yuo et al. (2009). Root hair growth stops after initiation in the mutant, resulting in reduced root and shoot growth. OsAPY protein shares a high identity with rice apyrase-like proteins (Yuo et al., 2009). The fact that the *Osapy* mutant has a high concentration of ATP indicates that reduced apyrase activity partly contributes to the resulting phenotypes. Over-expression of

apyrase genes from pea in *Arabidopsis* showed enhanced phosphate transport although no root hair morphology examination could be found (Thomas et al., 1999).

Another gene related to rice root hair growth, *OsCSLD*, belongs to a Cellulose Synthase-Like D1 Gene group (Kim et al., 2007). The functioning of *OsCSLD1* is required for root hair elongation but not initiation and the gene is expressed only in H cells. The homologous *Arabidopsis* gene *AtCSLD3* is expressed in root hair cells and is also found to have a function in root hair elongation, suggesting that rice and *Arabidopsis* share part of the root hair morphogenesis network (Kim et al., 2007). Other genes related to rice root hair growth include *OsPHR2*, which is involved in phosphate-signalling pathways (Wu and Wang, 2008), and *RH2*, which is likely to be involved in the Auxin pathway (Suzuki et al., 2003). However, the details about their effects on root hair growth and genes themselves are still lacking.

1.5 The effect of phosphate on root hair growth

Phosphate (Pi), which is the least available, highly immobile and non-renewable plant nutrient, limits the crop production unless supplied as fertilizer (Lambers H 2006). There are also problems associated with the acquisition of Pi) from soil by plants, including very low diffusion coefficients (Schenk and Barber, 1979), and high P-fixing capacity of soils (Barber HA 1995). On average, plants only recover 20% of the phosphate present in fertilizer (Gilbert N 2009). The global reserve of inorganic P (mined rock phosphate) is estimated to run out by 2060 (Arno Rosemarin 2004). Therefore, understanding the mechanism of plants responding to low phosphate at cellular and molecular level in order to improve the efficiency of P usage is critical for crop sustainability.

Plant roots use a number of strategies and root traits to improve Pi use efficiency (Paez-Garcia et al., 2015). Variation of root structure in low Pi

condition, such as shallower root growth angles, enhanced adventitious roots and dispersion of lateral root, was adapted by plants to increase Pi uptake from the top soil (Lynch 2007). The formation of root cortical aerenchyma (RCA) is also thought to be one of adaptations by the plants in low Pi conditions, remobilizing phosphorus from root cortex to reduce the root respiration and nutrient content (Postma and Lynch, 2011).

In *Arabidopsis*, the typical traits of a response to phosphate starvation are the shortened primary root, extensive lateral roots growth and proliferated root hairs towards the apical root (Bates et al., 1996). Unlike *Arabidopsis*, primary root and adventitious root elongation in rice is stimulated slightly by phosphate starvation when rice is grown in hydroponic solution, although the response of root hairs is the same as that of *Arabidopsis* (Zhou et al., 2008). The rice plant growing in low phosphate has significantly smaller shoots due to phosphate starvation and therefore increased root to shoot mass ratio (Jia H., 2011).

Root diameter and root hair morphology can also change in low phosphate conditions. In a study by Ma et al (2001), *Arabidopsis* root diameter was found to increase by 9%, resulting in more cortical cells without changing the cross-sectional area of individual cortical cells. Around four more trichoplast cell files were generated, resulting in the around 30% decrease of cross-sectional area of individual trichoplasts and atrichoplasts (Ma et al., 2001). In low phosphorus availability conditions, *Arabidopsis* root hairs were found to grow three times longer and five times denser than those growing in high phosphorus conditions (Bates et al, 1996, Ma et al., 2001).

In low phosphorus conditions, *Arabidopsis* plants were found to develop root hair length 3 times greater than that of plants growing in high phosphorus condition (Bates et al, 1996). To study the role of root hairs in phosphate uptake from soil, Gahoonia and Nielsen (1997) developed a method to separate the root hairs from root surface so the amount of

phosphorus taken up by root hairs only can be determined (Gahoonia and Nielsen, 1997). This study suggested that 70% of the root hairs growing in the radioactive phosphate labelled soil were responsible for 63% of the total phosphate uptake. In crop breeding program, selecting longer root hairs can often achieve more shoot phosphate content, hence more phosphate uptake from the soil (Gahoonia et al., 1998)

The plant hormones auxin and ethylene were also thought to be involved in root hair signalling pathway in low phosphate conditions. The auxin antagonist CMPA inhibited root hair elongation plants growing in low Pi conditions and low phosphorus stimulated root hairs elongation in the hairless *axr2* mutant, suggesting that auxin is involved in the plants' low phosphorus response in root hairs (Bates and Lynth, 1996). Ethylene is also thought to be involved in this response as root hair intensity was found to increase significantly under both high and low phosphorus availability with the addition of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid ACC (Zhang et al., 2003). The proportion of epidermal cells forming hairs and root hair length was reduced in ethylene-insensitive mutants, especially in the presence of low phosphorus in *Arabidopsis* which showed reduced response to low phosphate, suggesting its involvement in root hair responses to phosphorus deficiency.

Root hair density can be inhibited by adding ethylene inhibitors to low phosphorus media (Zhang et al., 2003). By contrast, under high phosphorus, average root hair density was found to be only marginally affected by the presence of ethylene inhibitors (He et al., 2005). Cortical cell number was not affected by ACC, or mutations reducing ethylene sensitivity in roots grown with low phosphorus, indicating that ethylene does not participate in this response (Zhang et al., 2003).

There is evidence suggesting that root hairs do not contribute to phosphate uptake in hydroponic solution culture. By using root the hairless mutant *rh2* and its wild type control rice cultivar Oochikara,

Suzuki et al. (2003) were not able to detect the difference in the amount of phosphate uptake per unit root dry weight (Suzuki et al., 2003). Research carried out using *Arabidopsis* varieties and mutants with contrasting root hair morphology by Bates and Lynch (2000a, b), and Gahoonia and Nielsen (1997) also suggested similar conclusions.

Root hair contribution to phosphate uptake has found to be undetectable under high phosphate soil conditions. The root hairless maize mutant *rth2* and *rth3* grown vigorously in normal soil conditions (Wen et al., 1994). However, their growth was reduced when plants were growing in soil with low phosphate (Wen et al., 1994). Compared with root hairless mutants *rhd6* and *rhd2*, wild type *Arabidopsis* plants showed greater biomass and phosphate uptake under low phosphate availability conditions in sand (Bates and Lynch 2000 a, b).

1.6 The *OsRHL1* bHLH transcriptional factor regulates rice root hair growth

A novel transcription factor with a basic helix-loop-helix (bHLH) domain was found to be involved in rice root hair development in an Indica rice species, *KAS* (Ding et al., 2009). With no significant difference in root length or the number of lateral roots and adventitious root, the mutant transgenic lines showed very short root hairs but similar diameters to normal (Figure1.2 B). *OsRHL1* over-expression transgenic lines (*RHL1-ov*) develop longer root hairs and were found to be able to thrive on low-nutrient media (Figure1.2 D) (unpublished data from Zhenjiang University). *OsRHL1* expression was detected only in nuclei and can be found specifically in root hair cells (Fig 2E, F). The fact that there is a loss of size difference between H and N cells in the mutant suggests that *OsRHL1* plays a role in the epidermal patterning network. These findings suggest that *OsRHL1* may control root hair elongation as well as root epidermal patterning.

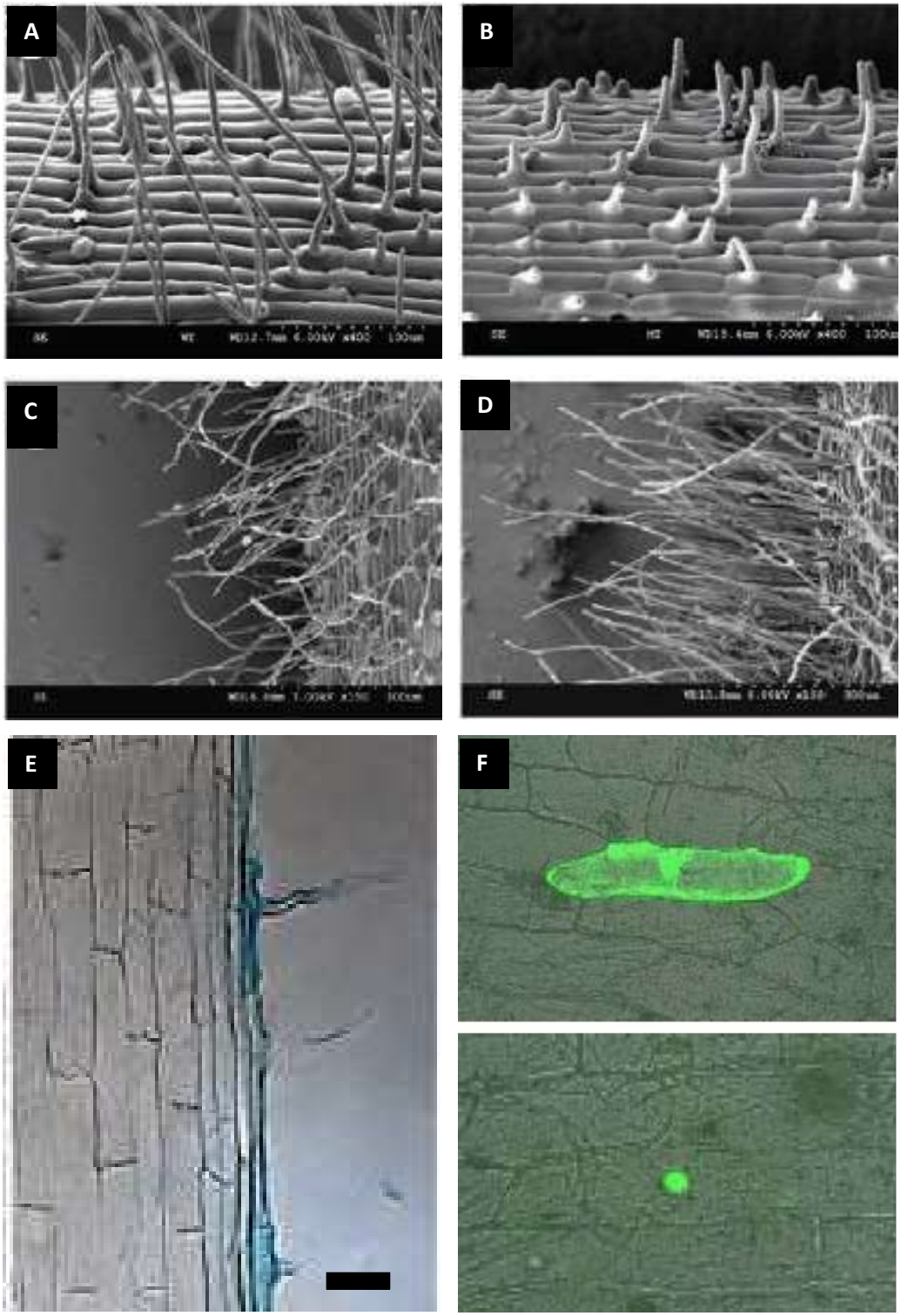


Figure 1.2: Cryo-SEM images of root hairs at 3 mm from root tip of WT (A) and rhl1-1 mutant (B). Cryo-SEM images of 2 to 3 mm from the root apex of the wild type (C) and overexpression of RHL1 (D). RHL1 promoter-driven GUS expression in roots. E, the longitudinal section of root hair region. Root hairs examined were in the 2 to 3 mm range from the root apex in a 100×100-μm2 region. *OsRHL1*::GUS reporter expression in longitudinal section of root hairs in wild type (E) and *OsRHL1*-GFP expression in nuclei of onion epidermal cells (F).

Genetic analysis suggests that *OsRHL1* gene belongs to subfamily C of the rice bHLH family and is highly homologous to members of subfamily 17 of the bHLH family in *Arabidopsis* (Ding et al., 2009). However, so far there is no evidence that proves that this family is involved in root hair development in *Arabidopsis* (Ding et al., 2009).

Analysis of the gene and its regulatory network will provide more information on rice root hair development, thus advancing our understanding of root hair development. Analysis of transcriptome data from root hair of *RHL1*-ov transgenic lines will allow us to identify differentially expressed genes which could contribute to the observed phenotypes and understanding the molecular mechanism underlying them.

Comparing the phosphate uptake using *OsRHL1* mutants (*rhl1*) and over-expression transgenic lines in low phosphate condition soil condition would also reveal further understanding.

1.7 Technology

1.7.1 Microarray and array data analysis

A microarray is a set of DNA sequences representing the entire set of genes of an organism, arranged in a grid pattern for use in genetic testing. There are many types of microarrays including DNA microarray. In DNA microarray, the DNA or RNA of cell, tissue and organ materials is labeled with the florescent. The density of every spot (a specific location for every gene sample, usually a gene fragment) is then measured to represent the expression level of that gene. It is a high-throughput and versatile technology used for parallel gene expression analysis for thousands of genes, allowing us to study genes within a genome at one time. It has been used widely by researchers as a gene expression profiling tool.

There are many microarray companies such as Affymetrix, Agilent, Applied Microarrays, Arrayit, Illumina and other one- or two coloured technologies. Affymetrix leads the way in large-scale production of DNA microarrays and has been widely used in the rice community. As the industry leader in the field of *in situ* oligonucleotide microarrays, Affymetrix further pioneered this technology by manufacturing GeneChips, its term for its high density oligonucleotide based DNA arrays.

The principle of manufacturing Affymetrix's Genechips is to manufacture short single strands of DNA onto 5-inch square quartx wafers and then the genes on the chip are designed based on this sequence. Using an industry chip synthesiser, sequences are then directly synthesised onto the surface of the wafer at selected positions.

Four major steps are usually required to perform a typical microarray experiment (i) preparation and labelling, (ii) hybridisation, (iii) washing, and (iv) image acquisition and data analysis. The first step is crucial as the overall success of the microarray experiment depends on the quality of the samples. The samples are prepared to isolate the total RNA at the time of sample collection. It contains messenger RNA that ideally represents a quantitative copy of genes expressed at that time. To further prepare the sample, mRNA extracted are then separately converted into complementary DNA (cDNA) using a reverse-transcriptase enzyme before being labelled with a tracking molecule, often fluorescent dyes.

The next step is to join the labeled cDNA to form a double-stranded molecule. This is then purified to remove contaminants such as unincorporated nucleotides, cellular proteins, primers, lipids and carbohydrates. The sample containing double stranded molecules is hybridised against cDNA molecules spotted on the genechip slides.

The slides are then washed after hybridisation. In this process, any labeled cDNA that did not hybridise on the array is removed. It also increases stringency of the experiment to reduce the possibility of cross hybridisation.

The final step of microarray experiments is image acquisition and data analysis. The image of the surface of the hybridised array is produced using laser scanner to determine how much labelled cDNA is bound to each target spot. The level of emission from each spot is measured using a confocal microscope.

There are several ways that Affymetrix use to reduce the possibility of cross hybridisation. A perfect match (PM) oligonucleotide and a mismatch (MM) oligonucleotide are used in each Affymetrix probe pair. The PM and MM probes are identical in sequence except for one single nucleotide. MM probes will hybridize to nonspecific sequences about as effectively as their counterpart PM probes. Moreover, instead of using one long sequence to represent one gene, multiple, short sequences are used to represent the unique sequence of genes. This PM-MM strategy, along with the multiple short sequences, helps identify and subtract nonspecific hybridization and background signals.

Microarray data analysis software

GeneSpring provides statistical tools suitable for the analysis of genomic structural variation and expression data. It has the advantage of providing a clear work flow and generating analysis results with good visual effect.

Another popular Microarray data analysis tool is Bioconductor which uses R programme. As an open source software, it benefits from extensive support from world-wide researchers and having advanced facilities for analysis of microarray platforms. The released Puma package from Bioconductor has the advantage of propagating

uncertainty from estimation of expression level to downstream analyses for improved microarray analysis (Al Pe 2008).

1.7.1.1 Microarray data normalization

A key issue when comparing DNA arrays is normalization, the process by which expression levels are made comparable between arrays. RMA (Robust Multi-array Analysis) is one of the methods used for normalization and summarization of microarray data to make them comparable (Naef, et al. 2003). There are three steps for this process. Firstly, the perfect match values combine background signal including optical signal, non-specific signal and a biological signal to be detected. To extract the biological signal, the perfect match values are subject to background correction in each individual chip which is based on the distribution of PM values amongst probes in an array in RMA. Secondly, a normalization procedure is performed to make chips comparable to each other. The method used for normalization in RMA is the quartile method, which assumes that the distribution of gene abundance is nearly the same in all samples. The pooled distribution of probes in all chips is taken as a reference chip and the new value for each probe in each chip is obtained from the reference chip at the same quartile. Finally, after background correction within each chip and normalization between different chips, the PM values are summarized to represent one single value from that probe set using a robust model fitting technique called median polishing to eliminate the effect of outlier probes.

The final expression value of each probe set representing each gene is decided using a baseline transformation method. The expression value of a gene in one chip is adjusted according to the mean or median of the values from different chips. The log ratio of the value for the gene is chosen to be its final representation in that chip. The median of the baseline transformation is chosen for the analysis.

1.7.1.2 Microarray data quality control

GeneSpring adopts three ways to evaluate the quality of the arrays, including: (i) 3'/5' ratio, (ii) hybridization control plots, and (iii) principal component analysis on samples. 3'/5' ratio is used to measure the efficiency of the cDNA synthesis by examining the ratio of expression intensity for the 3' probesets of housekeeping genes and those of 5'. A ratio value with more than 3 indicates degraded starting RNA in the sample or problems with the cDNA synthesis reaction.

Pre-mix hybridization control transcripts added in the samples in known concentration are used to monitor the hybridization and washing process. The profiles representing signal intensity of probes from hybridization controls in each sample are similar and show variable concentration of the probes.

1.7.2 Gene function annotation software

1.7.2.1 Harvest

Developed by the University of California, the HarvEST software is an EST database-viewing software in support of gene function analyses and oligonucleotide design. It is available for many organisms. For rice, it includes only the assembly that was produced by Affymetrix for the rice GeneChip. It contains best BLASTX hits from Uniprot (August 2008), BLASTN from rice (MSU version 6; January 2009), Brachypodium (Phytozome Bradi 1; May 2009) and BLASTX from *Arabidopsis* (TAIR version 9; June 2009) gene models.

1.7.2.2 Genevestigator

Genevestigator is an on-line platform which enables researchers to explore public and proprietary expression data globally. It stores a large database of manually curated and quality-controlled microarrays and RNAseq samples from a very large variety of tissues and conditions. Researchers can find out how genes are expressed in different tissues,

stimuli, diseases, drug treatments and genetic modifications. Database content for rice contains 130 studies, 2527 samples, 324 conditions, 368 genotypes and 38 anatomies.

The raw data is processed using quantile normalization method as implemented for RMA in Bioconductor. For comparability, the microarray expression values in Genevestigator are scaled so the average is equal to 1000 which gives a rough indication of the strength of expression.

Genevestigator uses the concept of meta-profiles for the expression value. It means that each signal value corresponds to the average expression level of that gene over a set of samples sharing the same biological context, e.g., samples from the same disease treatment. Genevestigator uses five types of meta-profiles to group samples according their anatomical parts, cell lines developmental stages, cancers types and perturbations types. All samples in the database are annotated according to these biological dimensions. An analysis based on meta-profiles assumes that sample expression values are comparable between the different experiments and between laboratories. However, for the perturbations meta-profile, data from multiple experiments are not mixed up, results are created by comparing groups of samples from individual experiments.

1.8 Aim and Objectives

OsRHL1 has been found to regulate the root hair growth in rice (Ding et al., 2009). *OsRHL1* expression can be further investigated using microarray transcriptomic data containing the expression profile of *OsRHL1*. The aim of this project is to use public and proprietary expression data together with microarray data from *RHL1*-ov transgenic line to study the function of *OsRHL1* gene.

The first objective is to check the expression profile of *OsRHL1* from publicly available database. To achieve this, the Gene Expression

Omnibus (GEO) and GeneVestigator will be first used to study these expression profiles by using their public and proprietary expression data. This will give an indication of where the gene *OsRHL1* is being expressed. The factors affecting the expression of *OsRHL1* include plant hormones and environmental factors, and change of expression of genes in the regulatory pathway where *OsRHL1* might be in. Genes co-regulated with *OsRHL1* can be also explored under the assumption that co-regulated genes means co-expressed genes responding with the common set of conditions and that are expressed in same type of tissues.

The second objective is to obtain the list of genes which are regulated by OsRHL1 to understand the gene regulatory network behind long root hair phenotype in OsRHL1 over-expression transgenic line. The microarray data from *RHL1*-ov transgenic line will be analysed by comparing with these from wild type to find groups of genes acting with *OsRHL1* to regulate root hair growth in rice. This result will be then compared with the mechanism of *Arabidopsis* root hair growth.

Another objective of the project is to validate whether the length of root hair in rice *OsRHL1* transgenic line has any effect on phosphate uptake. This will be achieved by using *OsRHL1* transgenic lines growing in hydroponic solution with variable concentration of phosphate. The growth parameters will be measured and analysed to decide the overall rice plant growth and plant phosphate content.

Food security is a challenge we are facing and studying how rice could take more phosphate from soils with no phosphate application will contribute to addressing this challenge. Rice root hair plays an important role in phosphate taking from soil. As *OsRHL1* is one of the key genes moderating root hair length, study collaborating these processes will contribute to the food security.

Chapter 2: Bioinformatics analysis of rice root hair growth related gene *OsRHL1*

2.1 Introduction

The root system performs essential functions during crop growth at vegetative, reproductive and ripening stages that include anchoring, water and nutrients uptake (Uga et al., 2015). As outgrowth of the root system, root hairs have always been thought to play important roles in enhancing the function of roots and therefore increasing crop's ability to take up more nutrients from the soil.

As one of the world's three major crops and the model species for the monocotyledonous plants, the rice root system has been a subject of intense study. However, comparing with well-studied *Arabidopsis* root hair development mechanism, the mechanism of rice root hair development is less well understood hence, more investigation Is needed to further understand the roles of rice root hair in its growth. *OsRHL1* has been found to play vital roles in rice root hair elongation and potentially the patterning and initiation process (Ding et al., 2008). Understanding the group of genes, and the environmental stimulus, interacting with *OsRHL1* will provide us with insight into the process of rice root hair development and therefore shed light on the potential application of genetic approaches to improve rice yield.

OsRHL1 is located in Chromosome 6 between 4,203,024 to 4,205,233bp {4,191,181 to 4,193,423 (from RAP-DB) or 4,192,207 to 4,194,416 bp (from MSU)}. *OsRHL1* is localized in nuclei and contains the bHLH domain, acting as transcriptional factor (Ding et al., 2008). Using its locus name LOC_Os06g08500.1 sequence information from MSU Osa1 Release 7 Annotation, the Genomic sequence length of *OsRHL1* is 2210 nucleotides and its CDS length is 1104 nucleotides.

The protein has a length of 367 amino acids according to Genebank protein ID BAD72512. The structure of the protein is Helix-loop-helix domain, a DNA-binding basic region with 60-100 amino acids, followed by two alpha-helices separated by a variable loop region, which was found in specific DNA-binding proteins that act as transcription factors (Ding et al., 2008).

The expression of *OsRHL1* was found in root hairs cells in root by Ding et al. (2008). There is no cross sectional images of GUS fusion with the coding region of the gene has been found to date suggesting its expression in other ground tissues. The expression was also found in the above ground tissues such as hull and base of panicle branches (Ding et al., 2008).

Overexpression of *OsRHL1* is associate with notably elongated root hairs. Whilst on the other hand, plants with no or little *OsRHL1* expression have much shorter root hairs. In this chapter, bioinformatic methods will be used to identify the group of genes contributing to the phenotype of *OsRHL1* overexpression and *Osrhl1* mutant. Firstly the GEO database is used to find out lists of gene modification, hormone treatment and environmental conditions regulating the expression of *OsRHL1*. Secondly, Genevestigor will be used to explore *OsRHL1* expression anatomy and lists of co-expressed genes with *OsRHL1* and the associated pathway if there is any.

The study of these lists will focus on the genes in three aspects, including:

(i) Which homologs have known functions in *Arabidopsis* root hair development pathway. If the list of genes contains any gene which is similar to *Arabidopsis* root hair growth, the two processes can then be compared with each other.

(ii) Any of the nutrient uptake related genes, which indicate a potential relationship between nutrient uptake and root hair length.

(iii) Any plant hormone related genes. In *Arabidopsis,* plant hormone level has been proved to play an important role in root hair development including patterning, initiation and growth (Bates and Lynth, 1996), such genes will be investigated to understand potential links.

This study also aims to identify the list of environmental conditions that regulate the expression of *OsRHL1* therefore contributing to the change of root hair phenotype in *OsRHL1* expression modified plants. From the list, these conditions related to nutrient stress will then be investigated.

Identifying the key genes interacting with *OsRHL1* will help us understand the mechanism of rice root hair growth and the environmental factors affecting this process. Finally, this study also investigate the effect of hormones on the expression of *OsRHL1*, in order to find out whether there is any interaction between hormones regulating root hair development pathway and the *OsRHL1* pathway.

2.2 Using GEO database to search for conditions affecting *OsRHL1* expression

2.2.1 Methods

The expression level of a gene can be searched using gene names under the gene profiles search tool from the Gene Expression Omnibus (GEO), a database repository of high throughput gene expression data and hybridization arrays, chips, microarrays. Many corresponding names including probe name or clone ID of one gene of interest from different gene chip technologies can be used. Table 2.1 lists the names of *OsRHL1* used in the search. The detailed names of this gene can be found from the China Rice Data Centre, Rice Genome Annotation Project, and the Rice Annotation Project Database. The

protein accession name of *OsRHL1* can be found in NCBI. From this record, the associated genome DNA sequence with number AP005919 is the sequence of chromo 6.

As the GEO database is part of NCBI and there is a lack of CDNA gene names corresponding to *OsRHL1* in the NCBI database, no expression level can be found using Genebank nucleotide accession number, protein accession number or RAP-DB ID of *OsRHL1.*

In the Rice Genome Annotation Project, Rice annotation database, the *OsRHL1*'s locus number is LOC_Os06g08500. Using this locus number, the corresponding probe or clone ID from different technologies used for the microarray data generation was found. The site used for the Affymetrix probe conversion is OryzaExpress. Table 2.1 was generated using *OsRHL1* locus name.

Table 2.1: List of probe names from different microarray technologies.

Name of technology	**Probe or Clone ID**	**Search result**
Affymetrix probe set	OsAffx.27510.2.S1_at	Yes
	OsAffx.27510.2.S1_x_at	
Agilent probe	No probe	
Rice Operon	OsIFCC009692	Yes
	OsIRUA005404	

In the process of finding the corresponding microarray ID names of *OsRHL1*, the Rice Oligonucleotide Array Database was also used. The database searches probe names from array technologies including Agilent, BGI/Yale, NSF. Both MSU and RAP ID name are used for searching from the GEO website. Only Affymetrix probe name of *OsRHL1* was found both sites.

There are often two names associated with *OsRHL1* Affymetrix probe name with or without _x. Without "_x" indicates that all of the probes in that probe set represent only that particular sequence. Only the expression profiles from probe name without _x will be used when both names are able to produce result.

2.2.2 Results and Disscusion

In general, the expression level of *OsRHL1* is low compared with average gene expression level in roots (Fig 2.1a) and seedlings of rice. The expression in stems and leaves is even lower using RT-PCR method used in GEO profiles. Similar results were reported in Ding et al. (2008). GEO search result from profile GDS2631 (not shown) indicates the expression of *OsRHL1* in rice coleoptiles and the expression appears not to be affected by anaerobic conditions the plant experienced. The expression of *OsRHL1* in rice leave samples from GEO profile GDS3441 (not shown) also suggests that anaerobic stress conditions do not regulate *OsRHL1* expression. GEO profile GDS3441 suggests that the expression of *OsRHL1* can be detected in the leaf samples and do not seem to be affected by blast fungus infection.

Using probe name OsAffx.27510.2.S1_at, there are eight microarray datasets found with a change of expression in *OsRHL1* in these datasets. As mentioned earlier, only datasets containing environmental conditions, nutrients and hormone related modifications are discussed here.

GEO dataset GDS1800 (Fig 2.1a) was obtained using rice roots samples. This experiment was performed to compare the expression profile of two rice varieties, Bala and Azucena, with various degrees of salt tolerance, with arsenate tolerant Bala (Norton al et., 2008). While the expression level of *OsRHL1* in Azucena slightly reduces, it increases more than four fold in the salt tolerant variety Bala, suggesting that *OsRHL1* might be involved in salt tolerance pathway. In *Arabidopsis*, Wang (2008) suggested the link between salt stress and root hairs development includes patterning and elongation (Wang et al., 2008). In rice, Liu et al. (2006) suggested that there is a connection between salt tolerance and root hair. Over-expression of heat shock transcriptional factor *OsHsfA7*, which has been found to regulate the expression of heat shock proteins plays important part in salt tolerance

pathway, resulting in short root hairs and lateral roots (Liu et al., 2013). This indicates that *OsRHL1* regulated root hair modification could also regulated by salinity at in the environment.

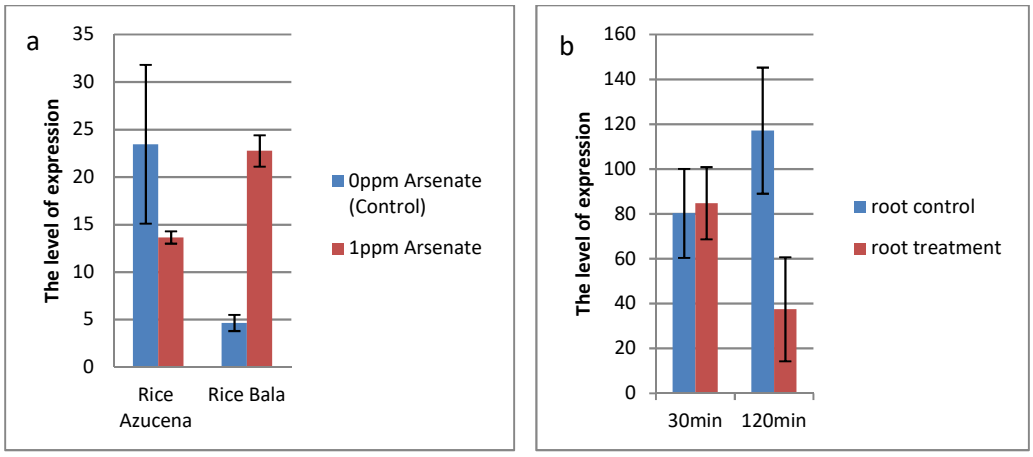


Figure 2.1: (a) The level of expression of *OsRHL1* at GEO dataset GDS1800, (b) The expression level of *OsRHL1* from GEO dataset GDS1798.The error bars represents the standard deviation of the corresponded data.

Cytokinin (CK) levels in root decreases when plants encounter stress (Hare et al., 1997). When plants grow in low phosphate condition, decreased level of CK stimulates the expression phosphate response genes through the CK receptor *CRE1* and *AHK3* and encourages the plant to respond to nutrient stress (Hirose et al., 2007; Franco-Zorrilla et al., 2005).

The search results from GEO also suggests that drought conditions affect the expression of *OsRHL1*. Using both *OsRHL1* rice operon IDs, OsIRUA005404 and OsIFCC009692 and taking the average expression value, the expression of *OsRHL1* in leaves was examined. *CBF3* in transgenic rice elevates tolerance to high salinity and drought while *ABF3* in transgenic rice increases tolerance to drought stress alone (Se-Jun Oh et al., 2005). Figure 2.1b suggests that the change of expression in *OsRHL1* is detected in the *ABF3* drought stress tolerance pathway rather than the pathway within related to *CBF* salinity and

drought tolerance. There is an increase in the *OsRHL1* expression in leaves when *ABF3* transgenic line suffers drought stress.

The expression profile GDS2632 in rice was compared between normal rice plants and other rice plants treated with CK trans-zeatin at two distinct time points. In the wild type control, the expression of *OsRHL1* in root was detectable from each of three replicates. However, after the root sample was treated with CK trans-zeatin to increase the level of CK in the rice plant, the expression level of *OsRHL1* becomes undetectable. Figure 2.2 showS that, in root samples, after 30 minutes of treatment, there is little change in *OsRHL1* expression. However, after 120 minutes of treatment, the difference between control and treatment is significant. The increased CK level in root after 120 minutes results in decreased level of *OsRHL1* expression in root without CK treatment. It suggests there is a negative relationship between the level of CK and the level of *OsRHL1* expression.

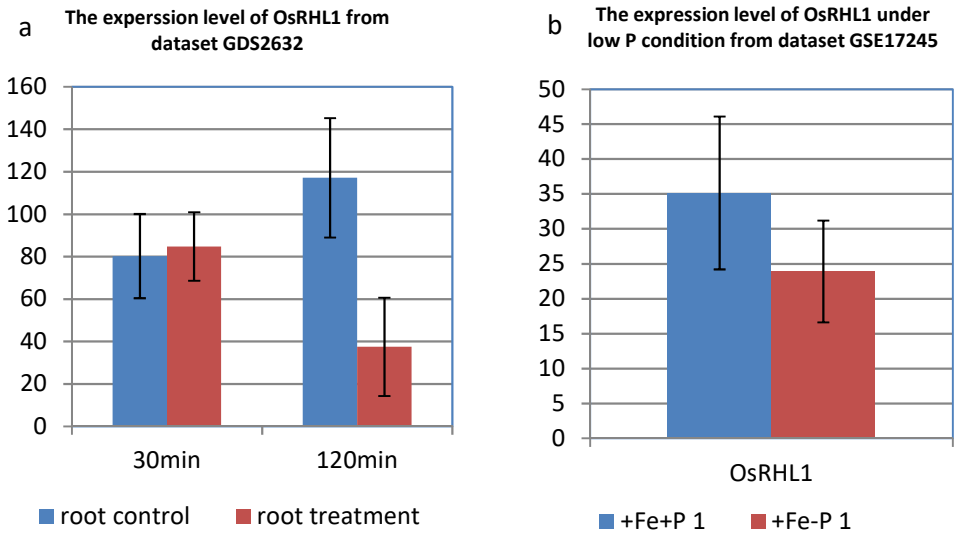


Figure 2.2: (a): The expression level of *OsRHL1* at GEO dataset GDS2632 at 30 mins and 120 mins after the CK treatment. (b): The expression level of *OsRHL1* at GEO dataset GSE17245 under saturated Fe and low phosphate condition. Error bars represents the standard deviation of the corresponded data.

Although the expression level of *OsRHL1* falls slightly under low phosphate conditions, statistical analysis indicates that this difference is not significant. The low phosphate level seemed to not affect the expression of *OsRHL1* in roots. This indicates that although high CK level in plants result in low expression of *OsRHL1*, the low CK level associated with phosphate stress does not result in high expression of *OsRHL1* in rice.

2.3 Bioinformatic analysis using GeneVestigator

2.3.1 GeneVestigator search method

Genevestigator (Hruz et al., 2008) was also used to investigate *OsRHL1*. The software utilizes thousands of publicly and privately curated datasets to find out where, when, and in what condition the studied gene is expressed. The concept of mega-profiles is used to summarize expression values for a given type of condition into representative expression values. This assumes, even though expression values from different experiment are incomparable, the representative expression values from all available datasets will help to understand the character of the gene.

The search begins by selecting an organism Oryza Sativa as the organism to study, using the sample selection. The Platform used in this organism is Affymatrix rice genome array. *OsRHL1* can be added to the search using gene selection. The *OsRHL1* gene can then be added by using its MAU name, RAPdb number and Genebank accession number apart from Gene symbol (Table 2.2). If probe set name for the gene is used, both numbers need to be added.

Table 2.2: The list of *OsRHL1* names used for GeneVestigator study.

Gene symbol	***OsRHL1***
MSU Locus Name	LOC_Os06g08500
Probe set name	OsAffx.27510.2.S1_at, OsAffx.27510.2.S1_x_at
RAPdb	Os06g0184000
Genebank accession number	BAD72512

To find co-regulated genes for *OsRHL1* using GeneVestigator, a list of co-expressed genes in response to a common subset of conditions are grouped in the same type of tissues.

Firstly, the conditions that regulate *OsRHL1* are considered. In GeneVestigator, after selecting organism rice and all available samples, the perturbation tool is used to find the conditions of interest. The conditions which *OsRHL1* is regulated are filtered using fold change larger than 1.5 and $p < 0.05$.

Secondly, using the co-expression tool, the top 25 genes that are co-expressed under these relevant conditions, whether having positive or negative relationships with *OsRHL1*, are generated.

Lastly, according to their expression across tissues, these genes are grouped together. A hierarchical clustering tool was used to cluster these genes by similarity across anatomical parts. The details of the list of genes can be seen from Appendix 1. The genes that are closely co-expressed with *OsRHL1* and expressed in roots were then subject to analysis. The function of genes was then found using Uniprot and Rice Genome Annotation Project.

2.3.2 GeneVestigator analysis results

2.3.2.1 *OsRHL1* expression anatomy results

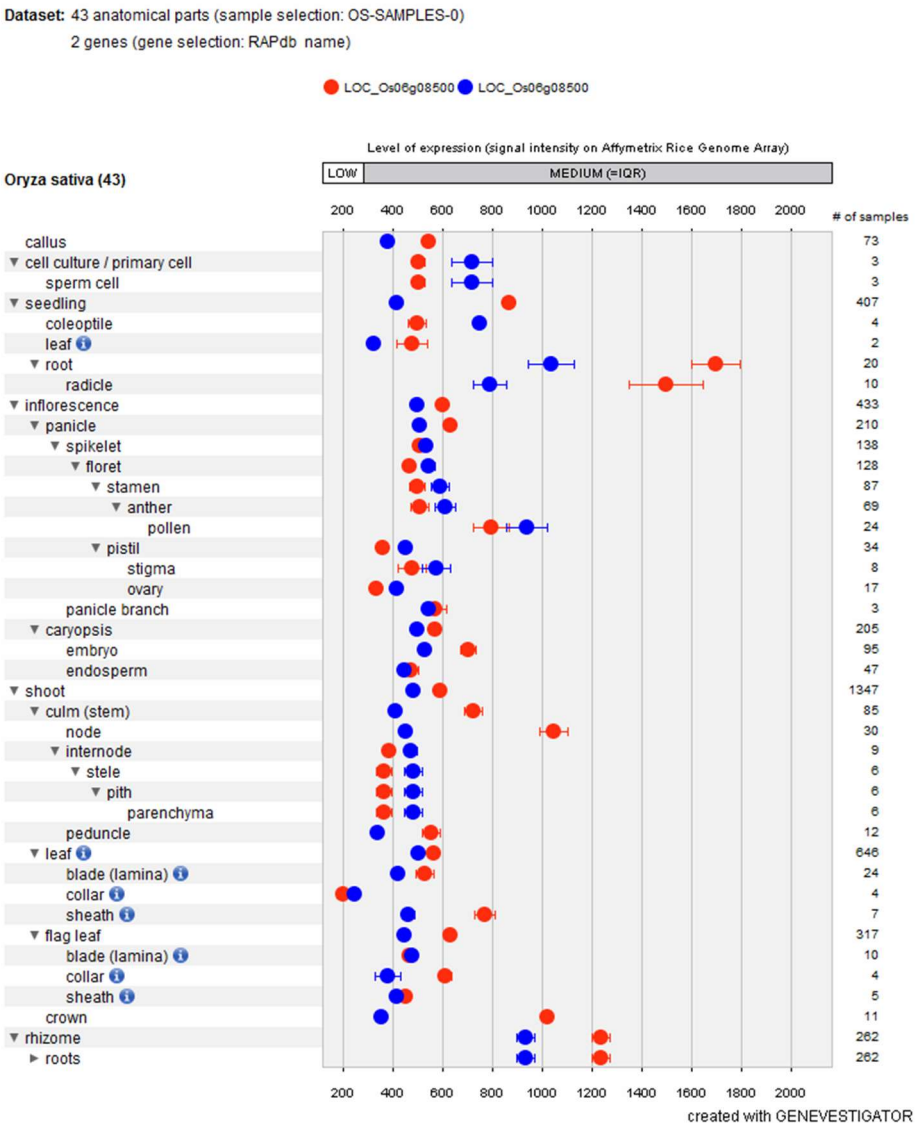


Figure 2.4: *OsRHL1* anatomy analysis result using GeneVestigator.red dot represents LOC_Os06G08500 while green dot represents LOC_Os06g08500.1

The GeneVestigator allows us to search the gene anatomy using gene expression datasets. From Figure 2.4, as Ding et al. (2008) suggested, the expression of *OsRHL1* is the strongest in rhizomes including seeding roots and seedling radicle. The expression level in caryopsis and peduncle, which is also detected in Ding et al. (2008) is slightly higher than the in the rest of the plant. From Figure 2.4, it can also be concluded that the expression of *OsRHL1* is higher in floral pollen. This

is not mentioned in Ding et al. (2008). For the rest of the plant, the expression is in the low to medium level and can't be detected.

2.3.2.2 Co-regulated genes result

There is a range of genes with different functions that are co-regulated with *OsRHL1* (Table 2.3). While some have no functions, some of these facilitate chemical reactions. LOC_Os01g71340, LOC_Os01g54700, LOC_Os08g21760, LOC_Os08g10350, LOC_Os04g35540 are located in membrane and have a transport function. LOC_Os08g21760, LOC_Os01g51700 are involved in Golgi transport and both have positive revelations with *OsRHL1*, suggesting that the long root hair phenotype in *OsRHL1* over-expression transgenic line might be the result of up-regulated transport genes at the tip of the root hair which is also the case in Arabidopsis tip growth (Grierson and Schiefelbein. 2002).

2.4 Conclusion

The bHLHs *OsRHL1* plays an important role in rice root hair development. The gene expression has been studied using conventional approaches (Ding et al., 2008). To further investigate the gene, transcriptomic data is used to further investigate the gene properties to answer question such as where and when the genes is expressed, under what circumstances and with which other genes. Data from GEO suggests that *OsRHL1* is involved in the salt tolerance pathway as the salt tolerant variety shows higher level of *OsRHL1* expression in roots after the plant is being treated with arsenate. The expression of *OsRHL1* occurs in rice coleoptiles and it appears unaffected by anaerobic conditions the plant experienced in. Results also suggest that the expression in the leaf does not change with blast fungus infection.

Table 2.3:Co-expressed genes with *OsRHL1* expressed in root.

Co-reguated with LOC_Os06g08500 in root	**revelation**	**Functions**
LOC_Os04g59000	Negative	Protein kinase family protein
LOC_Os02g09050	Negative	N/A
LOC_Os04g49194	Negative	Naringenin,2-oxoglutarate 3-dioxygenase, metal ion binding
LOC_Os01g71340	Negative	Glycosyl hydrolases family 17, anchored component of plasma membrane
LOC_Os02g42630	Negative	N/A
LOC_Os02g28074	Negative	XRN 5-3 exonuclease N-terminus family protein, nucleic acid binding
LOC_Os01g54700	Positive	Retrotransposon protein, integral component of membrane
LOC_Os08g21760	Positive	Rer1 protein, retrograde vesicle-mediated transport, Golgi to ER, integral component of membrane
LOC_Os01g53450	Positive	Aminotransferase, classes I and II, domain containing protein, catalytic activity
LOC_Os09g29780	Positive	N/A
LOC_Os02g46500	Positive	U-box, ubiquitin-protein transferase activity
LOC_Os01g51700	Positive	Ras-related protein, Golgi to endosome transport
LOC_Os06g33180	Positive	N/A
LOC_Os07g12220	Positive	N/A
LOC_Os08g10350	Positive	Integral membrane family protein
LOC_Os04g35540	Positive	Amino acid permease family protein,,L-amino acid transmembrane transporter activity
LOC_Os05g38120	Positive	Homeodomain protein, DNA bindin

The GEO study indicates that there is a relationship between *OsRHL1* expression and plant hormone CK. After 120 minutes of treatment using CK trans-zeatin, the expression of *OsRHL1* in roots decreases significantly. As the CK concentration is often related with environmental stresses including phosphate stress, the relationship between phosphate stress and *OsRHL1* expression is investigated using transcriptomic data. Results suggest that phosphate stress does not appear to affect the expression level of *OsRHL1* in roots. In leaves, the expression appears to be associated with *ABF3*-related drought tolerance pathway, which is elaborated in the *ABF3* transgenic line.

The study using GeneVestigator software took advantage of the considerable amount of transcriptomic data from public and proprietary sources. Its use of mega-profiles concept makes it possible to study

gene anatomy and identify co-expressed genes. The results of anatomy agree with those Ding et al. (2008)'s and indicate that expression level of *OsRHL1* is detected in roots, caryopsis and peduncle. It also appears in floral pollen which was not reported by Ding et al. (2008).

Using GeneVestigator, a list of co-regulated genes has been identified, suggesting *OsRHL1*'s functions with a group of genes related to transport and membrane location. This is related to its function in root hair development as the growth of root hair cells is involved in transporting the materials for cell wall growth and passing membrane for accumulating crucial substances at root tips for growth. The transcriptomic study is based on the gene expression data and each profile has a different error profile. Further RT-PCR study can be carried out to confirm the list and establish the network related to *OsRHL1*.

Chapter 3: Transcriptomic data analysis in *OsRHL1* transgenic lines

3.1 Introduction

Transgenic line over expressing *OsRHL1* indicate longer root hairs than wild type rice (Ding et al., 2008). The purpose of this study is to identify the genes which contribute to the phenotype showing in *RHL1-ov* and *rhl1*.

In *Arabidopsis*, the accumulation of ROS (reactive oxygen species) and high Ca^{2+} concentration at the tip of root hairs, regulated by *NADPH oxidase* and *ROP GTPase*, was found to be important in root hair initiation (Foreman et al., 2003; Jones et al., 2007). Root hair tip growth was maintained at the elongation stage by ER and actin cytoskeleton at root hair tips (Ishida et al., 2008). Auxin was also found to play a role in the organization of the actin and microtubule filaments (Wu et al., 2011). Genes that affect this process in the ER with actin and auxin accumulation would results in changes in root hair morphology (Ishida et al., 2008). The biosynthesis and composition of the plant cell wall are regulated by *XETs*, *UGE4*, *LRX1*, *OsCSLD1* is also part of the root hair growth process (Bernal *et al*., 2008, Ishida et al., 2008). In addition, plant hormones such as auxin and ethylene were also found to be involved in plant root hair development (Zhang *et al*., 2003, Bates et al., 1996). These hormones may act with low phosphate conditions to regulate this process (Zhang *et al*., 2003).

However, the process of *Arabidopsis* root hair development is known to be different from that of rice. In *Arabidopsis* the pattern is established in the embryo stage (Lin et al., 2001) while the pattern in rice appears to be generated randomly (Kim and Dolan 2011, Datta et al., 2011). In *Arabidopsis*, this pattern acts interactively with the plant hormones

auxin and ethylene (Niu *et al*., 2011) and it is position dependent (Berger et al., 1998a). There is no such evidence for rice. We still know relatively little about the process of root hair development in rice, although how this process is regulated in *Arabidopsis* is well understood.

The microarray data from root hair samples of both *RHL1*-ov transgenic line and wild type Kashala (*KAS*) rice were used for this study. By comparing the expression profile of over-expression transgenic line with its wild type control, the mechanism resulting in the long root hair length phenotype in over-expression transgenic line were investigated.

3.2 Materials and methods

Six original transcriptomic Affymetrix microarray expression files including three independent replicates of *OsRHL1-ov* transgenic lines and KAS wild type were provided by Professor Wu's group from Zhejiang University. Three replicates for root hair of wild type KAS (WT) are named KAS_2B_root_hair.cel (KAS.1), KAS_3_root_hair.cel (KAS.2) and KAS_root_hair.cel (KAS.3). Three replicates for root hair of *RHL1*-ov are named RHL1_over_2_root_hair.cel (RHL1.1), RHL1_over_3_root_hair.cel (RHL1.2) and RHL1_over_root_hair.cel (RHL1.3). Data files KAS.1, KAS.2, RHL1.1 and RHL1.2 ere performed together and files KAS.3 and RHL1.3 were carried out later to add up the number of replicates.

RNA and mRNA isolation for these samples was performed from roots of the samples. The Affymetrix rice gene chip was used, representing 57,381 genes in rice. These microarray data were subject to analysis using GeneSpring (Agilent) and Bioconductor including the Puma package.

The list of up-regulated and down-regulated genes in *RHL1*-ov transgenic line vs WT was generated by applying the RMA

normalisation method, and the intensity levels were baseline transformed into the median.

For the annotation of the differentially expressed genes, the file containing the list of Affymetrix probe set IDs was first searched against Rice Oligonuleotide Array Database (http://www.ricearray.org) for the corresponding MSU/TIGR locus number and RAP gene ID for each probe set. For the probe set IDs which do not have a corresponding TIGR locus number or RAP gene ID from the search above, the NetAffx Query from Affymetrix was used to search those IDs. The ID converter from RAP database was also used to find the missing ID number to expand the functional search for the same genes. MSU Rice Genome Annotation Database and Resource (RGAP) and Rice Annotation Project Database (RAP-DB) were used for the annotation of these genes. Also, the China Rice Data Centre Database (www. Ricedata.cn) has been used for further annotation and gene function analysis. EST database-viewing software HarvEST:RiceChip version 1.12 was used for gene annotation (http://harvest.ucr.edu/).

3.3 Results

As one of the ways for quality control of microarray expression data, PCA (Principal Component Analysis) allows to summarize the ways in which gene response vary under different conditions to expression data. Replicates from one experiment condition are supposed to group together while samples from different group are supposed to separate well. The two replicates that were carried out first time from each sample are grouped better while the remaining replicates that were carried out later are not grouping well with other replicates from the same sample (Figure 3.1).

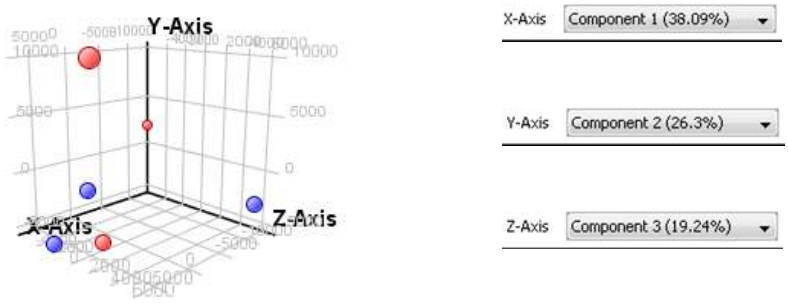


Figure 3.1: PCA plot produced using GeneSpring. The samples in red from left to right are KAS.1, KAS.3 and KAS.2. The samples in blue from left to right are RHL1.1, RHL1.2 and RHL1.3.

By using Bioconductor, the quality of the cell files can be visualized from (Figure 3.2). These image arrays suggested that, for unknown reasons, part of some arrays shows no hybridization signals or has very weak signal cluster compared with the rest of the arrays.

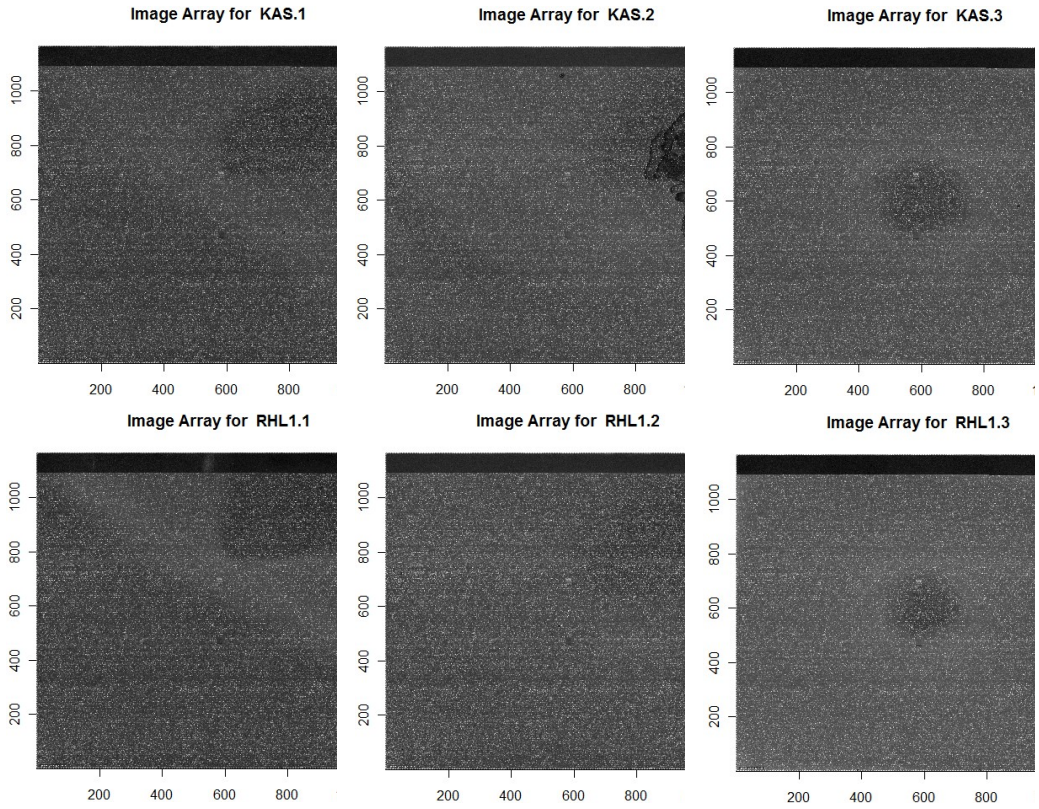


Figure 3.2: Array images of KAS.1, KAS.2, KAS.3, RHL1.1, RHL1.2, RHL1.3 produced using Bioconductor affy package. Each picture represents an image array for one sample with hybridization signal being missing or being very weak at some parts of the arrays.

The PMs are 11 to 20 oligos which are perfectly complementary to the mRNA of that gene. Their intensity distribution for all slides (Figure 3.3) indicates that 1 set of sample (*RHL1.3, KAS.3*) has higher level of expression profile. Two pairs of samples were collected at the same time while one pair of samples were collected at a later stage to add the number of replicates for the analysis. The PM intensity distribution histogram suggests that *RHL1.3, KAS.3* are those two samples which were performed later. It is also suggested from Figure 3.3 on the distribution of the sample *KAS.2* and *RHL1.2* that there are some uncertainties with the RNA quality of these samples.

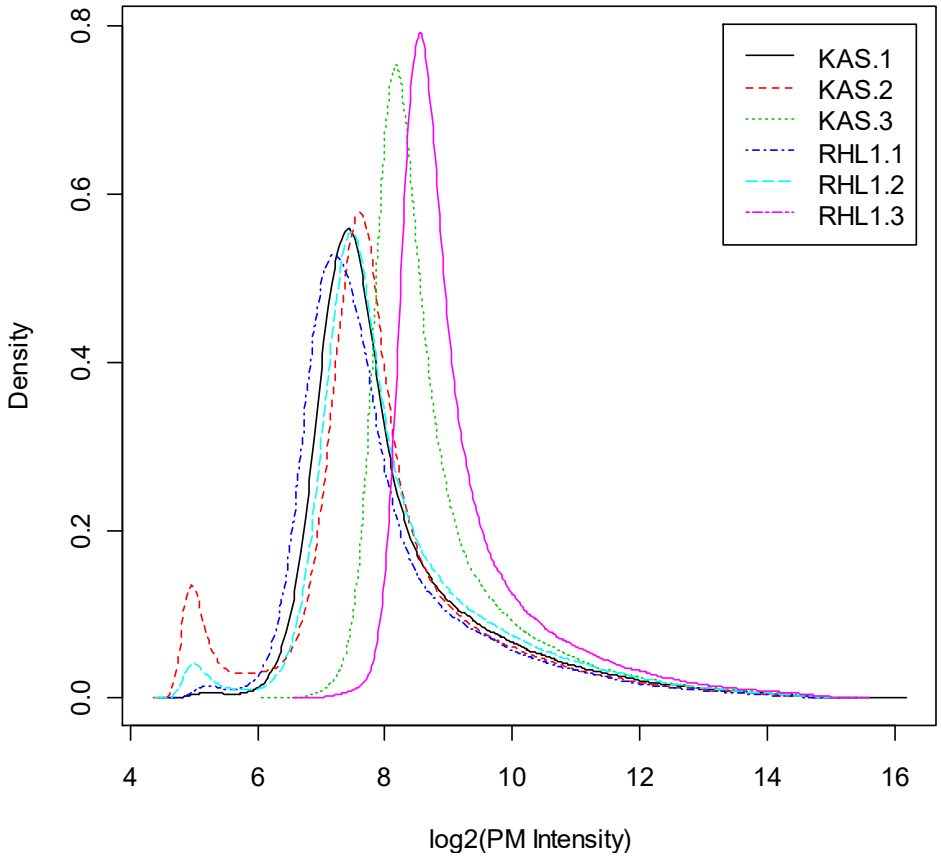


Figure 3.3: PM intensity plot for all slides generated with Bioconductor Affy package for sample KAS.1, KAS.2, KAS.3, RHL1.1, RHL1.2 and RHL1.3.

To check the quality of the samples which generated originally, analysis with different combinations of samples was performed using the

Bioconductor Puma package by plotting the PCA graph to see how well the samples are clustered for the two different treatments (over-expression and wild type). Figure 3.4 suggests that although two replicates from *OsRHL1*-ov performed at the first time (RHL1.1, RHL1.2) are grouped together better than others, two replicates from controls are still far apart. Further analysis and comparison were undertaken from the group with all six samples.

Although there are some variations between replicates from the same samples, after filtering out uncertain data and normalisation, the data is still valuable to purpose of analysis.

3.4 Differentially expressed genes between *OsRHL1*-ov transgenic line and wild type

Transcriptomic analysis using the Affymetrix rice array was performed to identify up-regulated and down-regulated genes from the *OsRHL1-ov* relative to the wild type control Indica rice cultivar Kasalath (*KAS*). Table 3.1 lists the probes for genes that are >2 times differentially expressed. These were separated at the basis of whether they were up or down regulated and were categorized by the biochemical or physiological role. Certain genes were found to have strikingly different transcript levels.

The transcriptomic data for *OsRHL1-ov* transgenic line was analysed to find the differentially expressed genes against the wild type. *OsRHL1-ov* transgenic line, the expression of OsRHL1 is 20 times more than in the wild type (Table 3.1). 43 genes were found to be up-regulated and 18 genes were down-regulated by *OsRHL1*. Nine genes including both up-regulated and down-regulated were found with known functions ranging from stress and disease resistance including *OsGLP8-2, rHb1, rHb2, OsHSP24.1*; promoting root and lateral root development such as NRR; cell division and elongation including *OsDSR-1, ADH1* and low phosphate response gene *OsSPX2*.

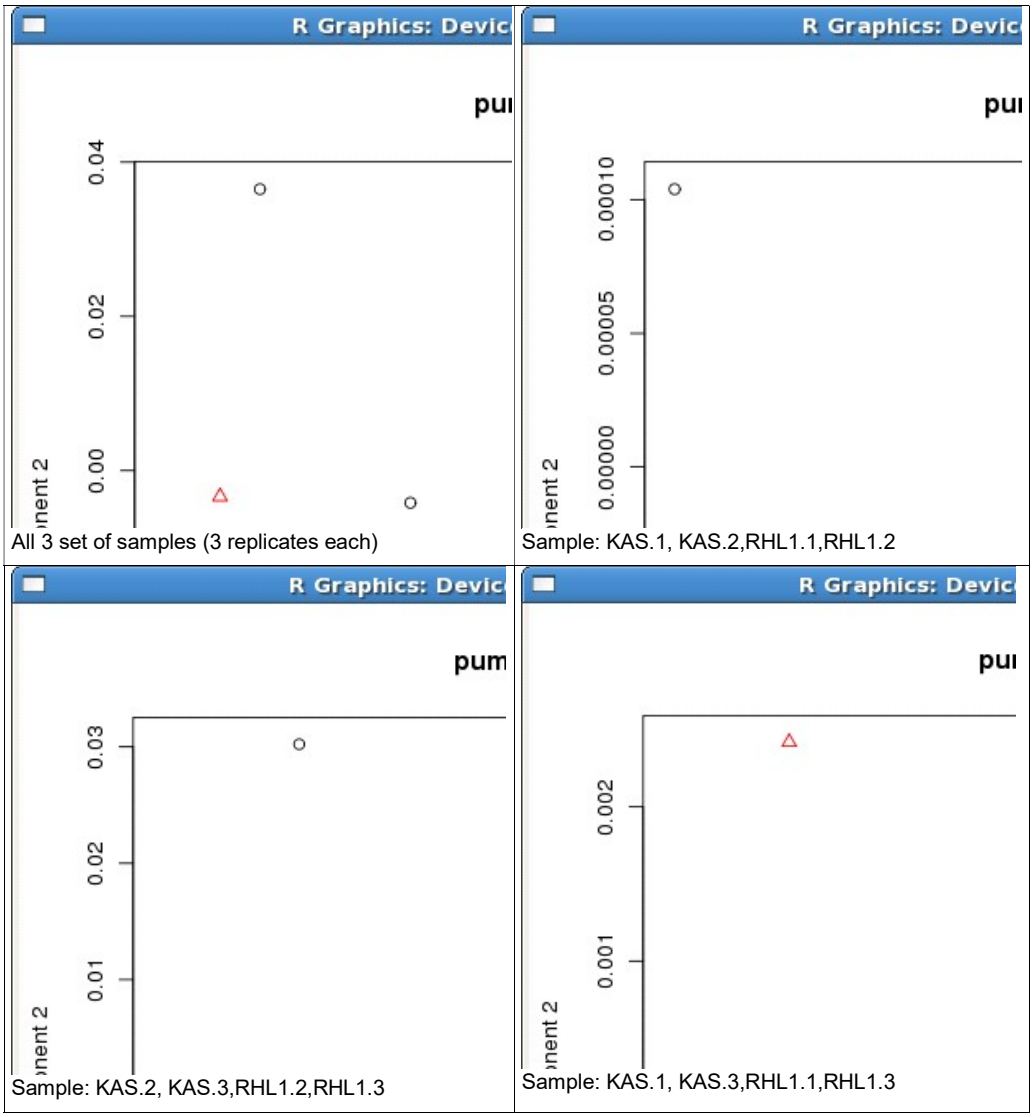


Figure 3.4: PCA plot from different combination of the samples using PUMA package from Biocondutor using samples KAS.1, KAS.2, KAS.3, RHL1.1, RHL1.2 and RHL1.3.

Seventeen were found with putative functions relating to stress, defence or disease response systems. Three genes including f-box containing LOC_Os10g41838, Myb with DNA-binding domain LOC_Os06g51260, WD40 containing LOC_Os10g35200 transcriptional factors were differentially expressed. Genes putatively involved in signalling process were also among those listed. These included a two-component response regulator and putatively genes involved in G proteins signalling pathway. One actin-related gene was found to be up-regulated in the over-expression transgenic line. Three genes from

the list were related to cell wall components which were found to be up-regulated in the transgenic line, suggesting that up-regulated root cell wall genes may contribute to the long root hair phonotype present in the over-expression transgenic line. Two genes were related to Ca^{2+} binding with approximately 3-fold change and also two genes were related to Oxygen binding. As one of the most down-regulated genes by *OsRHL1*, *OsSPX2* was involved in phosphate signalling network. The expression of *OsSPX2* was up-regulated in both root and shoots in low phosphate conditions (Wang Z et al., 2009). This was slightly down-regulated by the overexpression of *OsSPX1* and strongly by repression of *OsSPX1* (Wang Z et al., 2009).

Table 3.1: Differentially expressed genes between *OsRHL1* over expression and wild type control *KAS*

Gene structure and function	MSU/TIGR ID	Gene Symbol	Fold Change
Up			
Actin			
Actin-related protein 2/3 complex subunit 2, putative	LOC_Os01g46580		3.1
Binding			
hAT dimerisation domain containing protein, putative	LOC_Os01g36064		7.1
hAT dimerisation domain-containing protein, binding, putative	LOC_Os06g11830		7.1
hAT dimerisation domain-containing protein, putative	LOC_Os07g15340		7.1
VHS and GAT domain containing protein, putative	LOC_Os05g39760		2.0
Cell wall			
Retrotransposon protein, putative	LOC_Os07g32710		21.9
Cellulose synthase-like family E, putative	LOC_Os09g30120		2.6
UDP-glucuronosyl/UDP-glucosyltransferase family protein, putative	LOC_Os07g10190		2.3
Disease responsive			
Plant disease resistance response protein	LOC_Os07g44920		2.5
Rice Germin-Like Protein	LOC_Os08g08960	*OsGLP8-2*	2.3
GA hormone			
Gibberellin 20-oxidase activity, putative,	LOC_Os07g26100		17.4
Membrance binding			
Phosphatidylethanolamine-binding protein, putative	LOC_Os05g39250		4.1
P450-dependent fatty acid omega-hydroxylase, putative	LOC_Os01g63540		2.4
Metabolic process			
Amidase family protein, carbon-nitrogen ligase activity, putative	LOC_Os04g02780		4.5
UDP-glucuronosyl/UDP-glucosyltransferase family protein, putative	LOC_Os07g10190		4.4

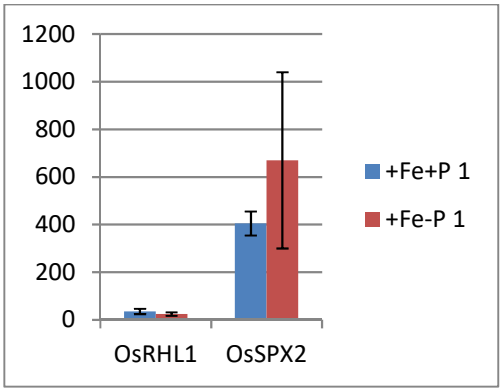
Uncategorized			
Similar to Mov34/MPN/PAD-1 family protein, putative	LOC_Os12g13674		11.1
Purine permease, putative	LOC_Os04g49757		7.1
Mitochondrial import inner membrane translocase subunit Tim17, putative	LOC_Os03g19290		3.2
CCT domain containing protein	LOC_Os05g51690	*NRR*	2.1
Similar to Alanine-glyoxylate aminotransferase-like protein, putative	LOC_Os05g39770		2.0
Signalling			
PLC-like phosphodiesterase, TIM beta/alpha-barrel domain containing protein.	LOC_Os03g40670		3.8
Ca+2 binding, EF hand family protein	LOC_Os10g09850	*OsDSR-1*	3.3
Conserved F-box and DUF295 domain,	LOC_Os01g47130		2.8
Tetratricopeptide repeat (TPR)-like superfamily protein, putative	LOC_Os06g40440		2.1
Two-component response regulator, putative	LOC_Os09g36220		2.1
Stress and defence related			
Seed maturation protein domain containing protein, putative	LOC_Os06g23350		5.3
Late embryogenesis abundant protein, putative	LOC_Os01g12580		4.8
Late embryogenesis abundant protein repeat containing protein, putative	LOC_Os01g50910		4.2
Dehydration stress-induced protein, putative	LOC_Os10g21790		3.8
Dehydrin, reponse to low temperatures and drought stress, putative	LOC_Os11g26780		3.7
Alcohol dehydrogenase gene	LOC_Os11g10480	*ADH1*	3.4
Caleosin related protein, similar to calcium binding EF-hand protein, putative	LOC_Os06g14324		3.0
GDSL-like lipase/acylhydrolase, involved in stress response, putative	LOC_Os02g50000		2.4
Pyruvate/Phosphoenolpyruvate kinase, involved in stress response, putaive	LOC_Os12g08760		2.4
Involved in stress tolerance mechnism	LOC_Os03g12510	*RHB2*	2.1
Involved in stress tolerance mechnism	LOC_Os03g13140	*RHB1*	2.1
Similar to Glutathione S-transferase GSTU31(GST 34), putative	LOC_Os10g38140		2.0
Trancriptinal factor			
bHLH domain,Involved in root hair elongation	LOC_Os06g08500	*OsRHL1*	19.1
F-box protein interaction domain containing protein, putative	LOC_Os10g41838		7.1
Unknown			
Hygromycin B phosphotransferase	N/A		644.0
Expressed protein	LOC_Os05g01330		20.0
Conserved hypothetical protein	LOC_Os03g51350		3.0
Conserved hypothetical protein	LOC_Os11g32890		2.7
Conserved hypothetical protein	N/A		2.6
Conserved hypothetical protein	LOC_Os08g41040		2.5
Conserved hypothetical protein	LOC_Os08g41070		2.4
Down			

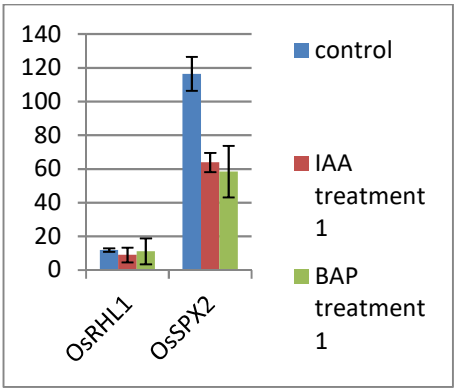
Sigalling			
SPX domain containing protein, involved in G protein associated signalling transduction	LOC_Os02g10780	*OsSPX2*	-11.9
Similar to Katanin p80 WD40-containing subunit B1, putative	LOC_Os10g35200		-2.8
WD, G-beta repeat domain containing protein, putative	LOC_Os05g33710		-2.6
Stress			
Heat shock dnaJ domain containing protein, putative	LOC_Os05g48810		-3.7
Heat shock protein Hsp20 domain containing protein, putative	LOC_Os06g14240		-3.5
Rice heat shock protein	LOC_Os02g52150	*OsHSP24.1*	-2.8
Similar to low molecular mass early light-inducible protein HV90, putative	LOC_Os01g14410		-2.7
Aldehyde dehydrogenase conserved site	LOC_Os03g14310		-2.3
Transriptional factors			
Myb, DNA-binding transcriptional factor, putative	LOC_Os06g51260		-2.5
Similar to Katanin p80 WD40-containing subunit B1, putative	LOC_Os10g35200		-2.3
Regulator of chromosome condensation, putative	LOC_Os05g38270		-2.1
Transporter			
Peptide transporter PTR2, putative	LOC_Os02g47090		-2.4
Heavy metal transport/detoxification protein domain containing protein.	LOC_Os01g61070		-2.4
Heavy metal transport/detoxification protein domain containing protein, putative	LOC_Os02g32814		-2.3
Unknown			
Conserved hypothetical protein	LOC_Os07g04450		-2.3
Expressed protein	LOC_Os06g03810		-2.2
Hypothetical protein	LOC_Os02g05670		-2.2
Conserved hypothetical protein	LOC_Os10g39500		-2.2

To further check the expression of *OsRHL1* in the effect of phosphate and building connection between *OsRHL1* and *OsSPX2*, the GEO expression dataset GSE17245 with expression profiles of roots of 10 days old rice seedling was extracted from different phosphate conditions.

Expression data from: (i) both *OsRHL1* and *OsSPX2* for full strength Fe, and (ii) either full strength phosphate or no phosphate were analysed to compare the effect of phosphate deficiency on rice plant root samples. The data were extracted from original affymetrix expression file and there are two replicates for each.

Figure 3.5a shows that lack of phosphate has little effect on the expression of *OsRHL1*. The expression of *OsSPX2* is increased significantly after the phosphate is taken away from the hydroponic solution.





(a) (b)

Figure 3.5 (a): Expression data of under the presence of phosphate and no phosphate in hydroponic solution from GEO dataset GSE17245. (b): expression data of both *OsRHL1* and OsSPX2 7-day old seedlings under the treatment of IAA and BAP. Error bar represents standard deviation of the corresponded data.

Plant hormone auxin and cytokinin was proved to sustain root hair growth by acting at the neighbour non-hair cells in *Arabidopsis* (Masucci and Schiefelbein 1994; Tanimoto et al., 1995). A synthetic form of auxin IAA and a synthetic form of cytokinin BAP are generally used to simulate the effect of these two hormones in experiment. To further investigate the effect of hormone treatment on the expression of *OsRHL1* and the relationship between the expression of *OsRHL1* and *OsSPX2*, the expression data of IAA and BAP treatment from GEO dataset GSE5167 (Figure 3.5b) was used. In general, the expression of *OsRHL1* is undetectable in these datasets. The standard error from the two replicates of each treatment is high therefore any effect of the treatment of the two plant hormones can not be confirmed. The effect of both hormones treatment to the expression of *OsSPX2* can be seen clearly and both reduce their expression. The relationship between *OsRHL1* and *OsSPX2* was not clear from the study.

3.5 Discussions and Conclusions

The transcriptomic data from the root hair sample of both *OsRHL1-ov* transgenic line and wild type *KAS* were used for the analysis. The PCA analysis generated using GeneSpring suggests that replicates from each of two samples are not grouping well, indicating variations in the replicates. The array images produced by Bioconductor suggest that a cluster of expression signal is missing or decreased from all but one sample. PM intensity analysis distribution produced using Bioconductor affy package further indicates the variation from the replicates. The analysis result gives us some indications of genes being regulated by *OsRHL1*. These results can then be confirmed by RT-PCR.

As an important gene in phosphate signalling pathway, *OsSPX2* is one of the most down-regulated genes in *OsRHL1*-ov transgenic line. *OsSPX2* is down-regulated in *OsRHL1* by 11-fold. From the same pathway, *OsPHR2* positively regulates the expression of *OsSPX1* (Wu and Wang 2008). Interestingly, the over-expression transgenic line of *OsPHR2* showed proliferated root hairs as seen in *OsRHL1* over-expression transgenic plant (Zhou et al., 2008). It would be interesting to build a connection between *OsRHL1* regulated and *OsPHR2* mediated root hair elongation pathways by examining the expression of *OsSPX2* and *OsRHL1* in *OsPHR2* over-expression transgenic line. One of the responses of rice to low phosphate is to grow longer root hairs. *OsSPX2* responds to lower phosphate by increasing its transcription level. However, in *OsRHL1* over-expression transgenic line with elongated root hair, a 10-fold reduction in expression of *OsSPX2* was found. This suggests that lower expression of *OsSPX2* does not result in better root hair growth. It could also indicate that *OsRHL1*-related root hair elongation pathway does not interact with phosphate mediated root hair growth pathway.

Among the genes that were differentially expressed, two of them were related to Ca^{2+} binding with around 3-fold change and two of them were

related to Oxygen binding. As Ca^{+2} and Reactive Oxygen Species (*ROS*) level is essential for the growth of root hair, upregulating these genes could potentially result in elongated root hairs seen in *OsRHL1*-ov transgenic line. *ROS* in root responds to nutrient deficiency and is associated with altered localization under phosphate starvation (Shin et al., 2005; Tyburski et al., 2009). Testing the concentration of *ROS* and Ca^{2+} in rhl1 mutant and the expression of *OsRHL1* in *rhd2* may further confirm their role and link to the *ROS* regulated P signalling pathway.

The differentially expressed list suggests a wide range of genes being involved in *OsRHL1* to achieve its long root hair phenotype in *OsRHL1*-ov transgenic line. The fact that actin and cell wall reproduction genes are found to be differentially regulated suggests that the mechanism of root hair growth between *Arabidopsis* and rice could be similar. *OsRHL1* also differentially regulates stress response genes, suggesting that *OsRHL1* could interact with the pathways of stress response by adjusting the root hair growth to adapt to different growth environments.

Ca^{2+} and oxygen binding genes are among the list of differentially expressed. These genes are found to be essential for the root hair tip growth in *Arabidopsis* and these findings indicate that mechanism of rice root hair growth is similar to that of *Arabidopsis*.

Although the root hair patterning mechanism is different between rice and *Arabidopsis*, the microarray analysis from *OsRHL1*-ov transgenic line indicates similarity in mechanism of root hair growth between *Arabidopsis* and rice, including root hair initiation and elongation.

Chapter 4: Phosphate uptake from hydroponic solution

4.1 Introduction

Phosphate is essential for crop growth and yield (Richardson et al., 2011). Typically the amount of phosphate present in natural soils is insufficient for optimal crop growth, therefore farmers rely on phosphorus fertilizers to maximise crop yield. However, present estimates are that plants use only 10–25 % of applied phosphate (Syers et al., 2008). Over-use of phosphorus fertilizers decreases phosphorus use efficiency (PUE), results in serious environmental consequences, and accelerates the depletion of phosphorus mineral reserves (Ashley et al., 2011). Knowledge of plant molecular mechanisms and architecture has the potential to improve PUE while maintaining environmental sustainability and preserving global food supplies (Lewis and Quirk 1967, Smith 2002, Husan et al., 2016). Some practical strategies to improve PUE include: (i) phosphorus mining and uptake using arbuscular mycorrhizal fungal symbioses (Berruti et al., 2015), (ii) achieving phosphorus activation and mobilization in the soil by intercropping with suitable crop species, (iii) tissue-specific over-expression of homologous genes for higher PUE, (iv) breeding for phosphorus-efficient varieties and introgression of key quantitative trait loci.

As one of the key quantitative trait, root structure plays an important role in increasing PUE. Plants responsed to low phosphate condition by altering their root architecture, including primary and lateral root length, root growth angle, root diameter and root hair morphology to maximize phosphate utilization. Root hairs alone are responsible for the majority of phosphate uptake from the soil by *Arabidopsis* (Gahoonia and Nielsen, 1997). Studies of root hair mutants of *Arabidopsis* suggest that root hairs facilitate phosphate uptake by increasing the absorptive surface area of the root at adjusting both root hair density and length

(Bates and Lynch, 2000b Peret et al., 2011). To date more than 40 genes are found to be involved in root hair initiation and development in *Arabidopsis*. The majority of them might relate to phosphate deficiency (Griersona and Schiefelbein 2002, Pe´ret et al., 2011). This phosphate concentration dependent root hair developments pathway might also involve other signal molecules including the plant hormones auxin, ethylene and cytokinin, and other signalling molecular such as reactive oxygen species and nitric oxide (Niu et al., 2013)

The experiment undertaken in this study is designed to measure the plant growth under various phosphate concentrations in hydroponic solutions. The three transgenic rice plants used for the experiment are *OsRHL1* over-expression (*RHL1-ov), OsRHL1* mutant *(rhl1),* wide type KAS plant (*WT*). They have distinctive root hair lengths. The length of root hair from *rhl1* is only 1/6 of its wild type while *RHL1-ov* has almost twice the root hair length (Ding et al., 2006). Apart from root hair length, there is no other root morphology difference in those transgenic lines. Therefore, they are ideal plant materials for studying the root hairs' response to phosphate concentration and uptake from hydroponic solution. By growing them in hydroponic solution with various concentrations of phosphate up to 60 days, growth parameters including shoot and root length, dry weight and the amount of phosphate in the plants can be measured.

This study aims to evaluate whether, in low phosphate condition, the transgenic lines with long root hair are able to uptake more phosphate from the hydroponic solution. The system successfully generated the phosphate deficiency environment for the plants. The growth parameters were then compared between transgenic lines to see whether the length of the root hair has any effect on plants growth. The focus is then switched to the phosphate concentration data obtained from dried plants shoot. The nutrient content of plant roots is then

compared and the limitations of the methods used and further improvement to the experiment are then discussed.

4.2 Methods

Seeds sterilization method

For germination on culture media, rice seeds were first surface sterilized in 10% NaOCl with 0.1% Triton X-100 and gently shaken for 30 minutes. The seeds were then rinsed with sterilized distilled water (SDW) several times. To prepare Petri dishes, a Stente Watman filter paper is cut into the shape and placed inside before adding 5ml SDW. Sterilized seeds were then placed in the petri dish, and then sealed with micropore tape (3M). The petri dishes containing sterilized seeds were placed in the growth room for approximately 4-7 days until the shoots began to appear. No sterilization was needed for seeds if they were grown in hydroponic solution.

Rice growing method in hydroponic solution

The hydroponic growth system was adapted from Erik Murchie's lab (Murchie EH et al., 2005). Germinated seeds were transferred from petri dishes to boxes with hydroponic solution. 1.5ml eppendorf tubes were used for the plants to grow after cutting the bottom ends and lids off with scissors (Figure 4.1). The tubes prepared were then placed in Piggyback tube rack (Sigma). Using tweezers, and each seed was carefully placed into the 1.5mL eppendorf tube with the root facing down the tube. Tube racks were then allowed to float on nutrient solution in a small bucket until leaf 1 is fully grown. When Leaf 1 is fully established, large black buckets full of nutrient solution were used to transfer plants to the white lids containing holes and sponges to hold the plants in place.

Figure 4.1: The procedure of rice growth in hydroponic solution. First, 7 days old rice plants are transformed in eppendorf tube growing into a basket of hydroponic water in growth room. And then plants are moved to larger basket in greenhouse.

Growth conditions

The growth room used for rice seed germination was maintained at 28 ^{o}C during 12 hours of light and 25 ^{o}C with 12 hour of darkness. Light intensity for the plant growth was about 90 umol $M^{-2}\ S^{-1}$. Plants were kept in the growth room for 7 days.

After the germination of the seeds, the seedlings were moved to glasshouse. 12 hours of light in 27^{o}C and 12 hours of darkness in 25^{o}C was maintained until the harvest which approximately takes 60 days.

Hydroponic solution formula

NH4NO3, 80.04mM: *NaH2PO4.2H2O*, 156.01mM: *K2SO4*, 174.26: *MgSO4.7H2O*, 246.47mM: *CaCl*2.2H2O, 147.01mM: *MnCl2.4H2O*, 197.91uM: *H3BO3*, 61.83uM: *(NH4)6Mo7O24.4H2O*, 1235.9uM: *ZnSO4.7H2O,* 287.56uM: *CuSO4.5H2O*, 249.69uM: *NaFe (3)-EDTA,* 367.05uM.

Plants materials

Plant materials used *OsRHL1* mutant (*rhl1*), *OsRHL1* over-expression (*RHL1-ov*) transgenic line, *KAS* wild type Indica rice (*WT*), provided by Zhenjiang University, China.

The hydroponic solution with sufficient phosphate (HP) contains 0.3mM of phosphorus. For low phosphate treatment (LP), 0.015mM was used in the solution. The plants were taken after 60 days of growth in hydroponic solution and shoot length was measured. Root length was determined using the average of the two longest roots. The plants were then oven dried and dry weight was measured afterwards. Dried plants were used for total P analysis by Yara.

In their lab, samples were prepared by ashing the samples in a high temperature oven at 540°C overnight and digesting the ash in HCl acid the following day. The sample was then filtered and analysed using a Varian ICPOES spectrometer. For sample preparation, the dried samples were milled and sieved. To weigh the samples for analysis, the milled sample was first stirred, and then the weight was taken.

Root nutrient analysis

Plant root samples were dried at 60 ºC for two days and then grinded in a stainless steel Wiley mill. Add 0.5g of dried sample and 5ml of concentrated nitric acid to a 50 mL Folin digestion tube. The mixture is heated to 120-130 ºC for 14 hours and is then treated with hydrogen peroxide. After digestion, the sample was diluted to 50mL and analyzed using ICP-MS from Scott Young's group for major, minor and trace components.

4.3 Results

4.3.1 The effectiveness of the system used

Here we assess whether the hydroponic system used is fit for purpose. Rice plants growing in low phosphate condition result in longer roots and shorter shoots (Figure 4.1). The mass of roots is slightly lower in low phosphorus conditions while the mass of shoot is significantly lower than those growing in sufficient phosphate condition. As a result, plants

in phosphorus stress condition weight significantly less in total than those under phosphorus sufficient condition (Figure 4.1).

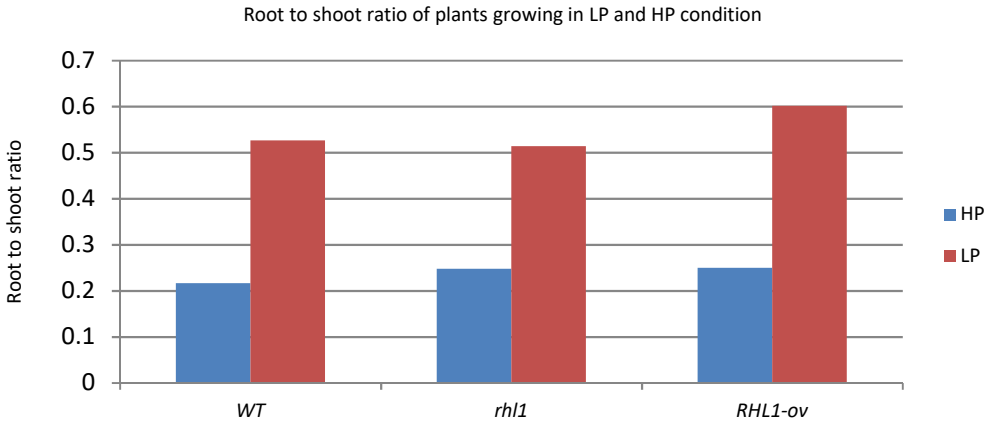


Figure 4.1: Root to shoot ratio of WT, *rhl1 and RHL1-ov* growing in HP and LP condition.

To test whether the system used here is able to generate the plants with the above characteristic, the growth parameters of plants were compared between each other. ANOVA test was used to indicate the difference of parameters caused by phosphate treatment. As predicted, *WT* plants grown in LP condition have shorter shoots as the result of lack of phosphate in the solution and longer root presumably by trying to reach more phosphate in the solution compared with these in HP conditions (Figure 4.2a,b). The difference between shoot length is statistically highly significant ($p<0.01$). While there is an apparent different in root length, this is not significant ($p>0.05$).

In terms of dry weight, plants growing in LP condition have much less total dry material than those in HP. Both parameters for shoot and total dry weight show a significant decrease in LP condition compared with HP. The largest difference in the dry weight occurs in the shoot of the plants. The dry weight of root is also much less in LP compared with these grown in HP condition although the length of root in LP is larger than those in HP. The same trends were detected across all three lines, *RHL1-ov, rhl1* and *WT* suggesting that regardless of the root hair length, for the same plants, those growing in LP condition have less dry weight

compared with those growing in HP condition. This is consistent with behaviour of the wild type plants growth in the same system (Figure 4.2).

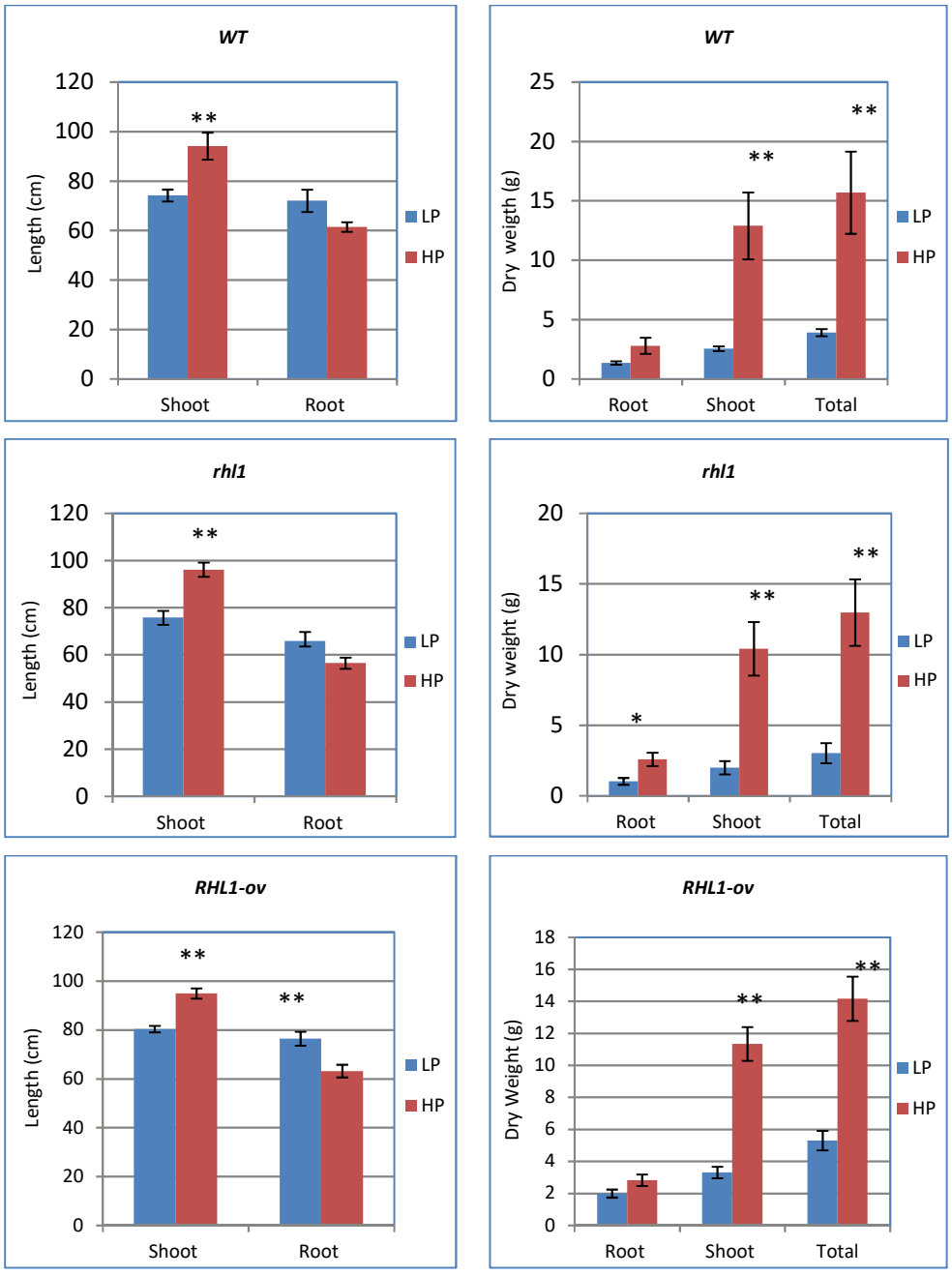


Figure 4.2: 60 days old rice WT, *rhl1 and RHL1-ov* plant roots, shoots length, roots, shoot and total dry weight at two different Pi levels (LP: 0.0015mM, HP: 0.6mM) in a hydroponic solution culture. Error bars represent SD (n=6).Star symbol suggest p value with one star representing <0.05 and two star <0.01

P-value from statistical significance test for the remaining growth parameters suggests that the difference in shoot length of plants growing between LP and HP condition is highly significant while the effect of phosphate concentration in the growth medium on root length and dry weight is not significant. Our conclusion is that the growth system effectively generates the LP plants (Figure 4.2).

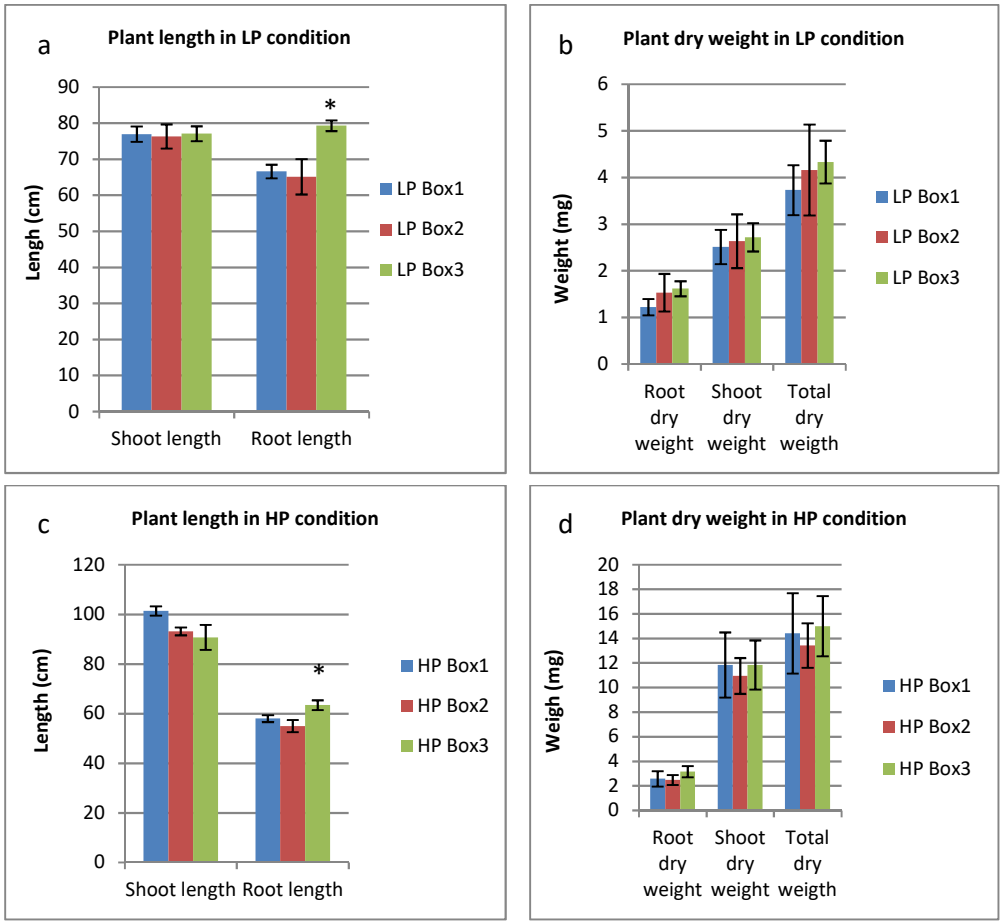


Figure 4.3: Comparison of plants growth from individual box containing hydroponic solution with a: plant length in LP b: plant dry weight in LP c: plant length in HP d: plant dry weight in HP condition. Error bars represent SD (n=6). Star symbol suggest p value with one star representing <0.05.

In phosphate deficient conditions, the root to shoot dry weight ratio of rice plants increases compared with plants growing under sufficient phosphate. To confirm that the experimental system used is able to produce phosphate deficient rice plants, the total root system of the

plants was analysed. As expected (Figs 4.1; 4.2), under phosphorus stress, root to shoot dry weight ratio of all plants increased.

When designing the system for hydroponic growth, the different plant lines used for the experiment were spread out in three different boxes and the number of each variety within each box is kept the same. By comparing growth of all plants between each box, it is expected that there will be no distinctive growth difference from each box.

Figure 4.3 shows that the difference of plant dry weight including shoot, root and total dry weight is not significant, suggesting that these parameters are comparable between each variety. However, in terms of root length, all plants from LP box 3 have longer root with $p<0.05$. In HP condition, HP box 3 has longer root and HP box 1 has longer shoot in all their plants with $p<0.05$.

4.3.2 Growth parameter comparison

After 60 days of growth at HP condition, the three lines used do not show any growth parameter differences (Fig 4.4).

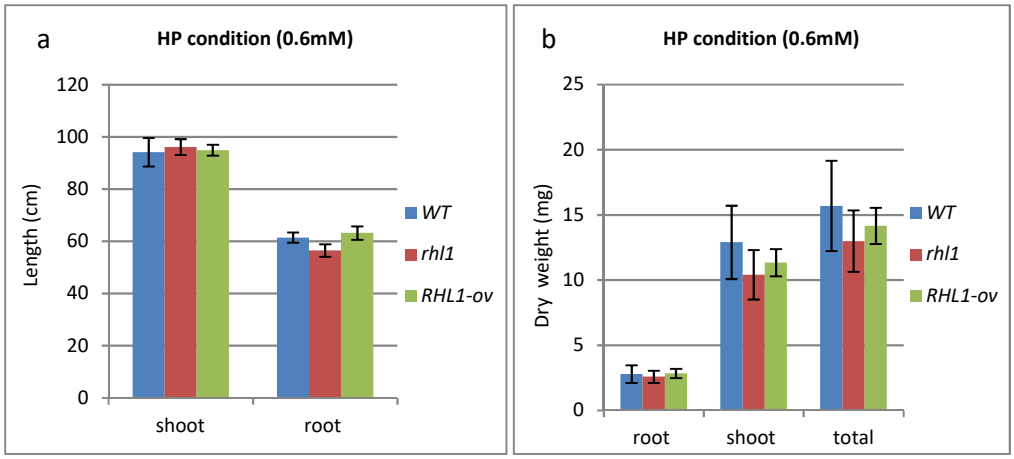


Figure 4.4: Growth performances of 60 days old seedling of wild-type (*WT*), *rhl1* mutant and *RHL1-ov* plants growing in HP (0.6mM) solution culture with a: plant length b: plant dry weight. Error bars represent SD (n=6).

Under low phosphate, data from Fig 4.5 suggests that in general, *RHL1-ov* grows better with longer shoot, root length and has more

shoot and root dry mass. Specifically shoot length and root dry weight of *RHL1*-ov have grown significantly better compared with *WT* (P<0.05). The growth parameters of *rhl1* appear to be shorter and smaller compared with those of *WT* apart from the shoot length measurement, but the difference is not significant.

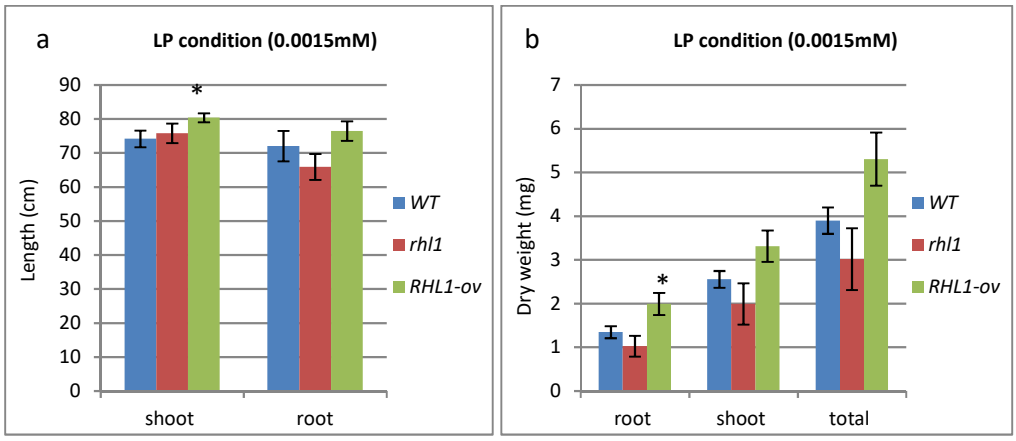


Figure 4.5: Growth performances of 60 days old seedling of wild-type (WT), *rhl1* mutant and *RHL1-ov* plants growing in LP (0.015mM) solution culture with a: shoot and root length b: dry weight. Error bars represent SD (n=6). Star symbol suggest p value with one star representing <0.05.

4.3.3 Plants shoot phosphate content comparison

On average, plants growing in HP solution have roughly 10 times higher shoot percentage concentration and 30 times higher total phosphate in shoot compared with those growing in LP condition (Fig 4.6). There is no significant difference between phosphate concentrations in shoot in LP condition between different lines. However, the amount of total phosphate content in shoot in *RHL1-ov* in low phosphate condition is significantly higher than these of the *WT*. The phosphate content of *rhl1* has no significant difference compared with *WT* in LP condition.

Meanwhile in HP condition, *RHL1-ov* has significantly higher phosphate concentration in shoot than *WT* while the total phosphate content is similar to *WT*. *rhl1* growth shows no significant difference in both average phosphate concentrations and total phosphate content.

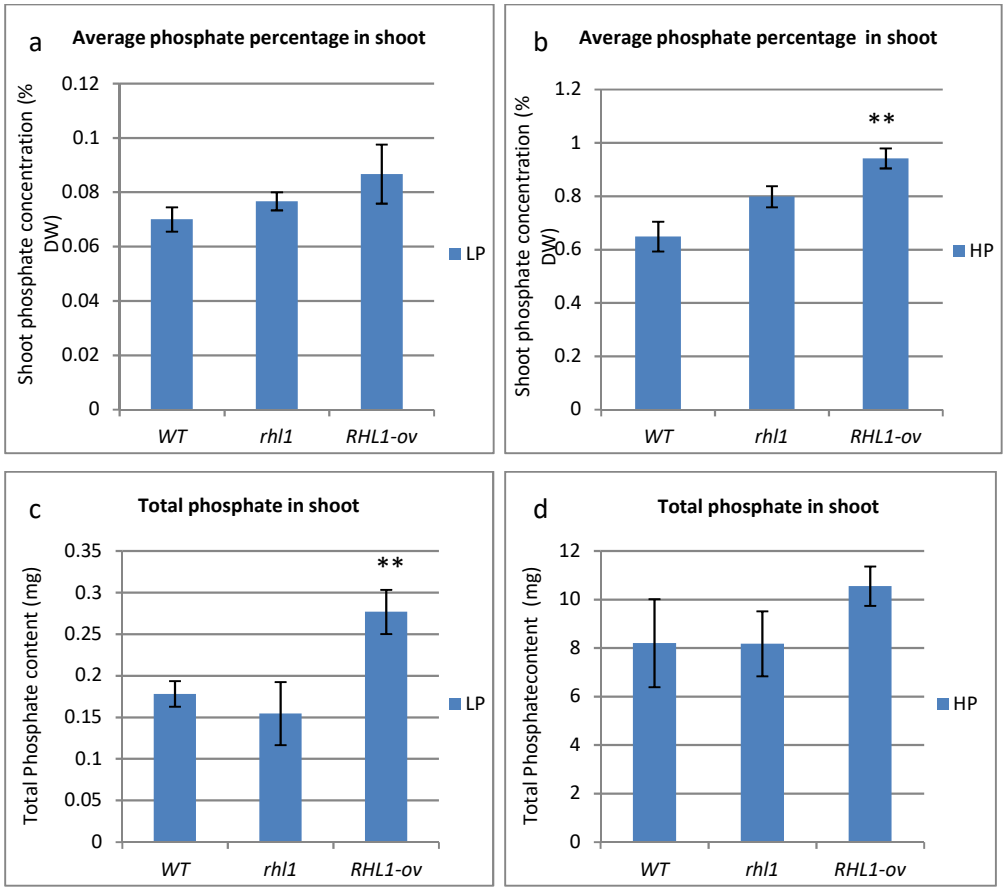


Figure 4.6: Shoot phosphate concentration in plants shoot with a: in LP condition b: in HP condition. Total phosphate content in shoot with c: in LP condition d: in HP condition. Error bars represent SD (n=6). Star symbol suggest p value with one star representing <0.05 and two stars <0.01.

4.3.4 Nutrient content in roots using ICP-MS

In general, roots growing in HP conditions have more nutrient content when compared with these growing in LP conditions, except for Potassium (K) (Figure 4.7). Roots from *rhl1* appear to have more nutrient concentration in HP conditions.

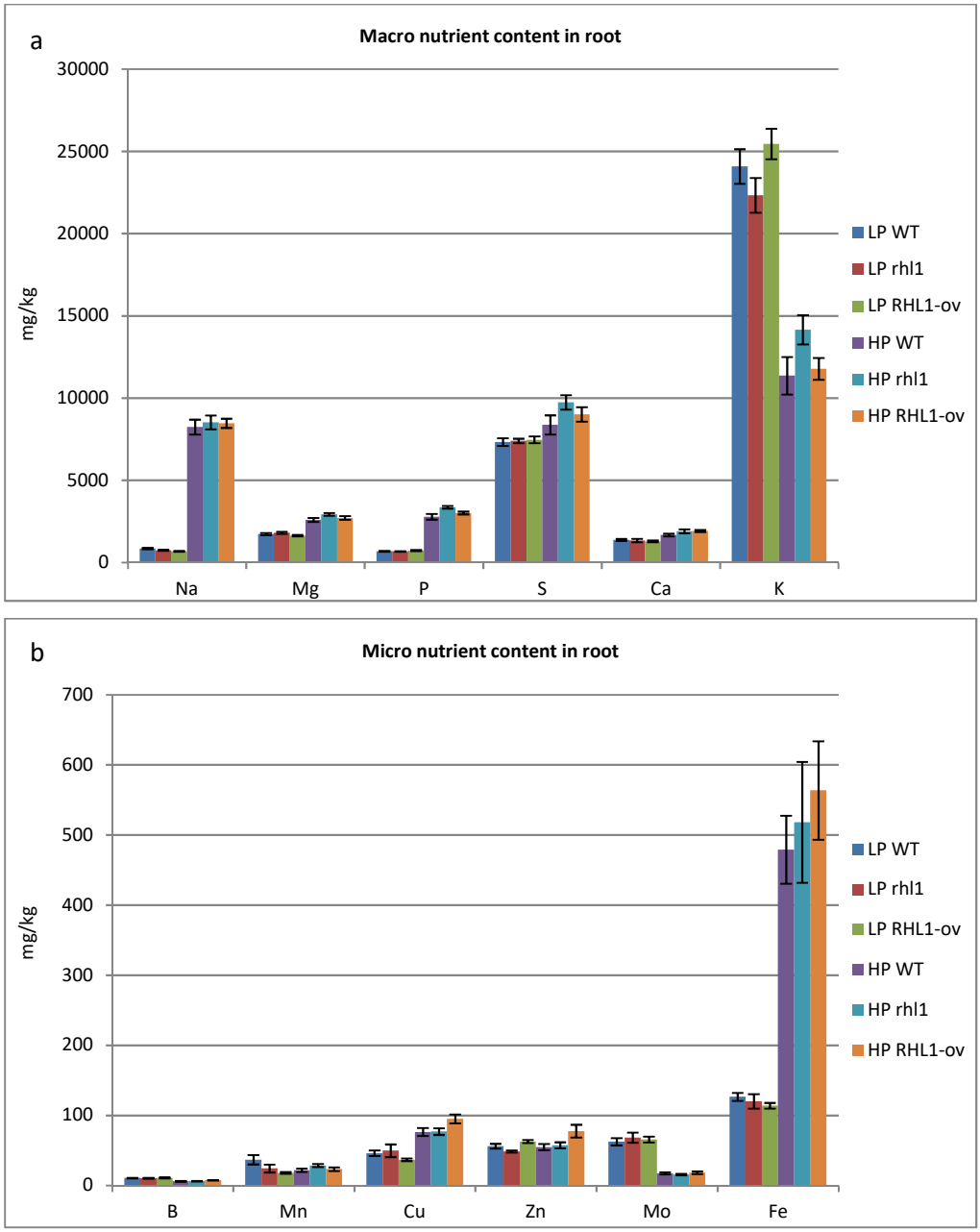


Figure 4.7: Plant roots nutrient content with a, macro nutrient b, micro nutrient. Error bars represent SD (n=6).

4.4 Discussion and Conclusion

To analyse the role of root hairs on phosphate uptake, three varieties with variable length of root hair were grown in hydroponic solution. The difference in growth parameters and phosphate uptakes between transgenic plants is assumed to be controlled primarily by the difference in the root hair length between them. The system successfully produce

plants characteristic of common phenotype difference between LP and HP condition (Figure 4.2). However by comparing the growth parameter from the each individual box, the root length is longer LP box 3. All plants in HP box 3 show longer root and HP box 1 showing longer shoot. There are a few factors which could contribute the difference showing here. The distribution of light intensity in the glass house could be uneven affecting the growth. The pH condition of each box is also one of the contributing factors affecting the growth. Although the hydroponic solution and its pH value is renewed and checked every 3 days during the period of growth, sometimes difference in pH value can be seen on the 3rd day when it is due to be checked. Having all plants growing in big box could limit this error.

In hydroponic solutions where there is sufficient phosphate available for the root hair to uptake, there is no significant difference in plant shoot, root length and dry weight between these lines. It suggests that root hair length does not play role in plant grow when phosphate is readily available (Figure 4).

Under LP conditions, *RHL1-ov* (longer root hairs) had significantly more root mass and longer shoot compared with *WT* (normal length of root hair) and *rhl1* (short root hair) (Figure 4.2). As Ding (2009) suggested, apart from the root hair morphology, there is no other phenotype difference between these lines. The difference in shoot length and root dry weight is the result of difference in root hair length. This suggests that length of root hair plays a role in plant growth in hydroponic solution when phosphate is not readily available. However the shorter hairs in *rhl1* did not result in less plant growth (Figure 4.2).

To analyse whether the better growth contributed by longer root hair is the result of greater uptake of phosphate to the shoot, the total phosphate in shoot was measured. The *RHL1-ov* plant growing in low phosphate condition has more total phosphate in their shoot compared with *rhl1* and *WT*. This indicates that better growth in *RHL1-ov* with

longer root hair is achieved through more phosphate in the shoot of these plants. It further suggests that plants with longer root hairs are able to take up more phosphate from low phosphate solution and contribute to better plant overall growth. However, *rhl1* (shorter root hair) does not show less plant shoot phosphate content as predicted.

When phosphate is limited in the growth media, the plants tend to grow more root presumably trying to locate phosphate in the solution and the root to shoot ratio increases (Figure 4.1). Compared with *rhl1* and *WT*, *RHL1-ov* are able to grow more root suggesting *OsRHL1* could interact with the genes controlling root growth under LP conditions.

Although in Ding et al., 2008, the root hairs length of *RHL1-ov* and *rhl1* were measured, this experiment did not measure the root hair length in HP and LP condition. Such measuring especially under LP conditions will help us to examine further the root hairs behaviour and contribution of them to the phosphate uptake.

Phosphate content analysis suggests that plants with long hair do not have higher phosphate concentration in shoot in phosphate stressed conditions although the total phosphate content is higher in these plants. However, this trend is not present in plants growing in solution with sufficient amount of P in it except that *RHL1-ov* has higher percentage of phosphate compared with the rest, but not significantly (Figure 4.6). The short hair from *rhl1* does not seem to affect its phosphate uptake from either LP or HP.

A number of attempts have been made to repeat this experiment in soil conditions. The soil formula used for soil experiment is from Glasshouse Crop Research Institute general purpose compost. The formula contains 25L Silver sand (from J Authun Bowers) 75L Sphagnum peat (From Shamrock) 10g NH_4NO_3, 150g KNO_3 250g Ground limestone 250g Magnesium Lime stone 40g fritted trace elements in 100L compost. Single superphosphate was used to provide P in the soil and 1.5g per litre provides P for the normal growth.

However, the rice failed to grow and became yellow after 5-6 days even under conditions with the normal amounts of phosphate. Erik Murchie later suggested that rice does not grow well in the soil formula with peat in. Sphagnum peat, also named peat moss, used is a popular growth media storing water. Information on rice performance on peat varies considerably. There are cases that rice do not grow or grow poorly (Kanapathy 1975, Dent 1980). There are also cases which rice grow really well on peat (Polak 1948, Noorsyamsi 1975). Peat fertilization is also site specific with no fertilizer application from Noorsyamsi 1975 to rice suffering nutritional disorders from Driessen (1978). Some other materials used as peat include coco peat and people experiencing the similar problem growing rice can be found using coco peat on internet. Suresh K Malhotra, from University of Southern Queensland suggested that the most abundant element of K (potassium) and salt may initially inhibit root development and lead to yellowing. The test on the level of K and salt from the formula used for rice growth can then be performed to eliminate these factors.

Chapter 5: Conclusions

OsRHL1 is a bHLHs transcriptional factor regulating root hair growth in rice. The over-expression of the gene results in longer root hairs in transgenic line while the mutant line has very short root hairs. To further study its property, GEO and GeneVestigator were firstly used to extract transcriptomic data of *OsRHL1* from microarray experiments. The comparison between the expression profile from *OsRHL1* over-expression transgenic line and wild type was used to identify the list of differentially expressed genes which could contribute to the long root hair phenotype in the over-expression transgenic line. The hypothesis of longer root hair resulting in more phosphate uptake was tested by growing over-expression transgenic line with longer root hair in hydroponic solution.

The GEO and GeneVestigator anatomy results agree with Ding et al. (2008) GUS analysis. The expression of the gene can be found in roots including seedling roots and seedlings radical, above-ground parts including caryopsis and peduncle. Results also suggest its expression in floral pollen which was not reported by Ding et al. (2008). The expression level also appeared higher in other parts of the plant such as sperm cell, culm, node and sheath. Expression data from up to 1347 samples were used and the expression value was for the given type of tissue. The method used assumes that the representative value close to the true value if the extraction was made from a large bundle of samples from the sample pool. As the number of samples for each tissue type varies considerably, the anatomy data is only an estimation and care should be taken when interpreting the results.

The GEO results from individual datasets suggest several links exist between the expression of *OsRHL1* and other environmental and hormonal factors. *OsRHL1* was found to be involved in the salt tolerance pathway of rice variety Bala and the drought tolerance ABF3

related pathway. Its expression was also suppressed by plant hormone CK treatment after 120 minutes. While the link between phosphate availability and CK concentration in plants is established, this study was not able to establish links between *OsRHL1* expression and phosphate availability.

A list of co-regulated genes was generated using GeneVestigator. The concept of co-regulated genes is to find the co-expressed genes in response to a common subset of conditions grouped in the same type of tissues. As *OsRHL1* is mainly expressed in roots, the type of tissues where the co-regulated genes were expressed was focused on roots. The list contains a number of genes with unknown functions and genes with transporting and membrane locating properties. Two Golgi transporting genes appeared in the list, further suggesting that rice acts in a similar way as *Arabidopsis* in terms of root hair growth mechanism, especially root hair tip growth (Table 3.1).

The microarray expression data for *OsRHL1-ov* transgenic line was analysed to investigate the list of differentially expressed genes contributing to the long root hair phenotype appeared in the over-expression line. Genes related to the cell wall production, root hair growth materials transport, other transcriptional factors and stress response are identified from the list (Table 3.1). This indicates that *OsRHL1* is co-regulated with other genes from the list, contributing to the long root hair growth in the over-expression transgenic line. Future work could be directed towards using a RT-PCR approach to confirm the microarray analysis results.

In the over-expression transgenic line the expression of *OsRHL1* was increased in laboratory approaches. The differentially expressed genes reflect the consequences of increased expression of *OsRHL1*. The analysis would be completed if microarray data from the *rhl1* mutant is available for comparison. *rhl1* mutant where the *OsRHL1* gene

expression is silenced has short root hairs and the list of differentially expressed genes will help to explain this phenotype.

The list from microarray analysis suggests that the genes were regulated by *OsRHL1*. It would be interesting to identify those genes or hormones that regulate the expression of *OsRHL1*. Recently, the LOTUS JAPONICUS ROOTHAIRLESS1-LIKE (*LRL*) proteins in *Arabidopsis* was found to be controlled by GLABRA2 (*GL2*) (Liang et al., 2014). *GL2* is a key gene in *Arabidopsis* root epidermal patterning network and it directly suppresses Basic Helix-Loop-Helix transcription factor genes including LRL (homologous of *OsRHL1* in *Arabidopsis*). Plant hormones was also found to regulate bHLHs gene. For example, Auxin was found in the *Physcomitrella patens* to promote hair growth (Tam et al., 2015).

To investigate whether long root hairs in transgenic line contributes to phosphate uptake from growth medium, the *OsRHL1*-ov transgenic line with long root hair, the *rhl1* mutant with short root hairs and *KAS* wild type as a control were grown in hydroponic solution. The result indicates that, in hydroponic solution with enough phosphate available, there is no significant difference detected in the growth parameters between long root hair *OsRHL1*-ov transgenic line, short hair rhl1 mutant and wild type *KAS* (Figure 4.6). However, when the plants were in low phosphate hydroponic conditions where the phosphate is less available for plant roots to take, the length of root hairs appeared to play an important role in phosphate uptake from hydroponic medium. *OsRHL1* over-expression transgenic line has long shoot length and it has significantly more root dry weight. The phosphate content in the shoot *OsRHL1*-ov transgenic line also have more average amount of total phosphate and subsequently more average phosphate percentage in shoot.

The conclusion that short root hairs contributes to poorer phosphate uptake can not be made using result from the *rhl1* mutant. In the low

phosphate, *rhl1* appears to have short root length, less shoot and root dry weight compared with wild type KAS control and over-expression transgenic line. However, this change is not significant. There are also no changes in terms of average shoot total phosphate amount and average phosphate percentage in the rhl1 mutant line.

The hydroponic rice growth experiment results suggest that *OsRHL1*-ov transgenic line with long root hairs growing in low phosphate conditions has greater total phosphate uptake. Further research could be undertaken to understand whether this is the case when plants are growing in soil conditions. In hydroponic growth, the phosphate is in the form of liquid and available for root hair uptake. However, this is not the case in soil. The phosphate is only available in rhizosphere and root hairs will not have more phosphate available to take once that rhizome is depleted.

Overall, the work shows *OsRHL1* plays a central role in root hair in response to the phosphate environment of the root. The long hair phenotype observed in *OsRHL1* over expression transgenic lines is able to take up more phosphate and results in more phosphate content in shoot. Salinity and phosphate conditions affect the expression of *OsRHL1* and the expression of *OsRHL1* is co-regulated with a number of genes and hormones related to root hair tip growth. Further study in *OsRHL1* will help us understand the gene network regulating rice root hair growth and its relationship with phosphate in the environment therefore increasing phosphate uptake.

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Appendix 1:

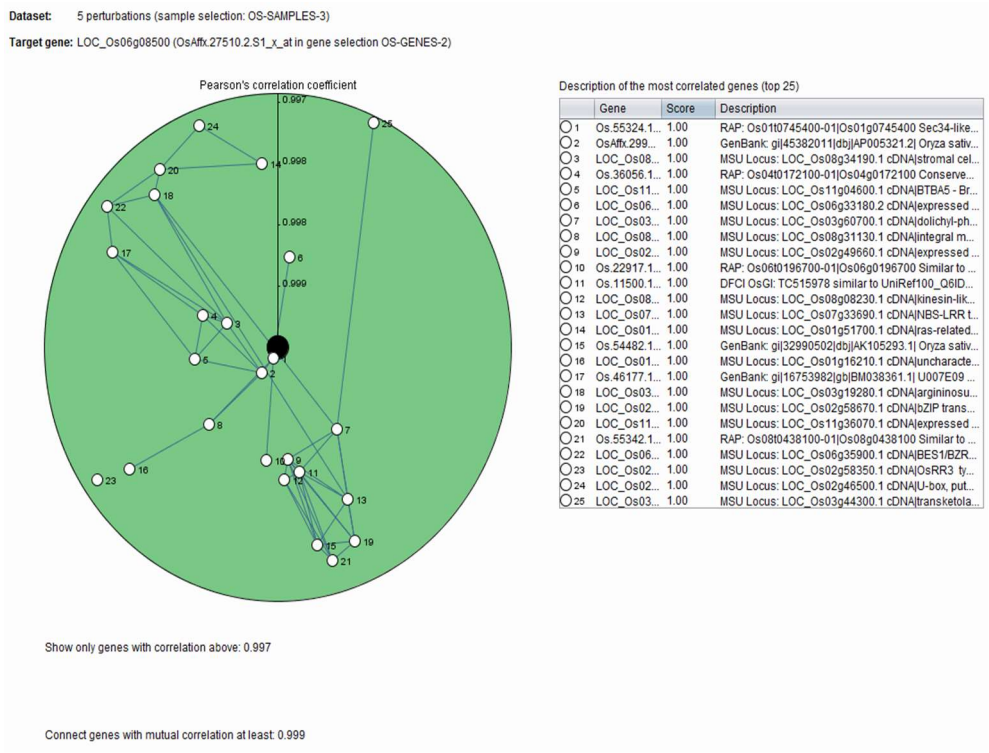

Figure 2.5: Top up-regulated 25 co-expressed genes with *OsRHL1* generated using GeneVestigator

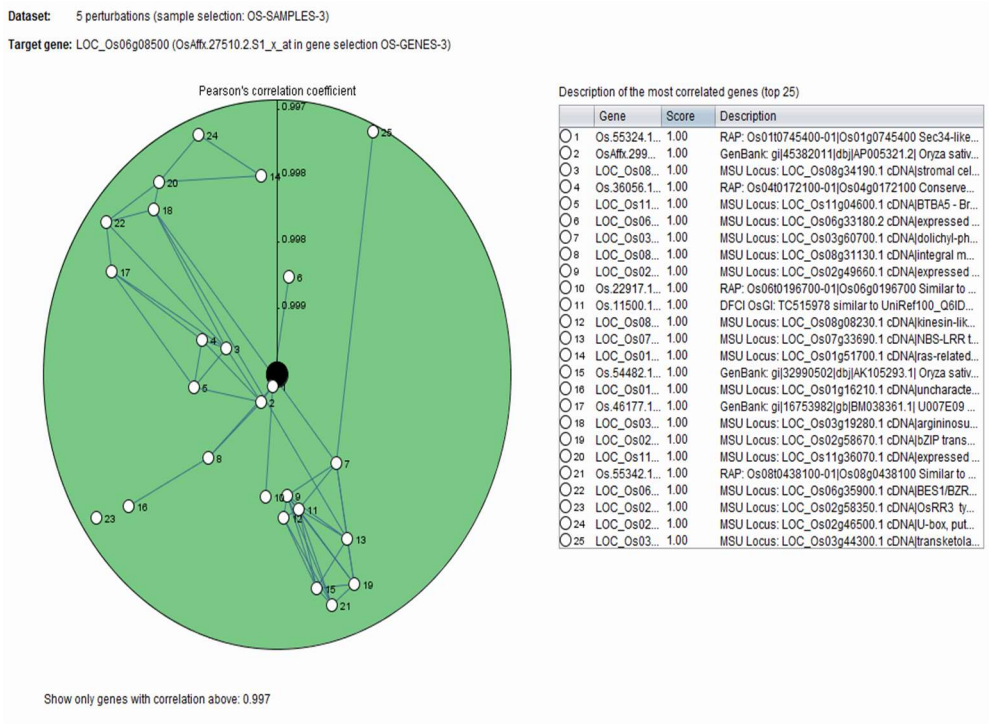

Figure 2.6: Top down-regulated 25 co-expressed genes with *OsRHL1* generated using GeneVestigator

Figure 2.7: Hierarchical Clustering result for down-regulated genes generated using GeneVestigator

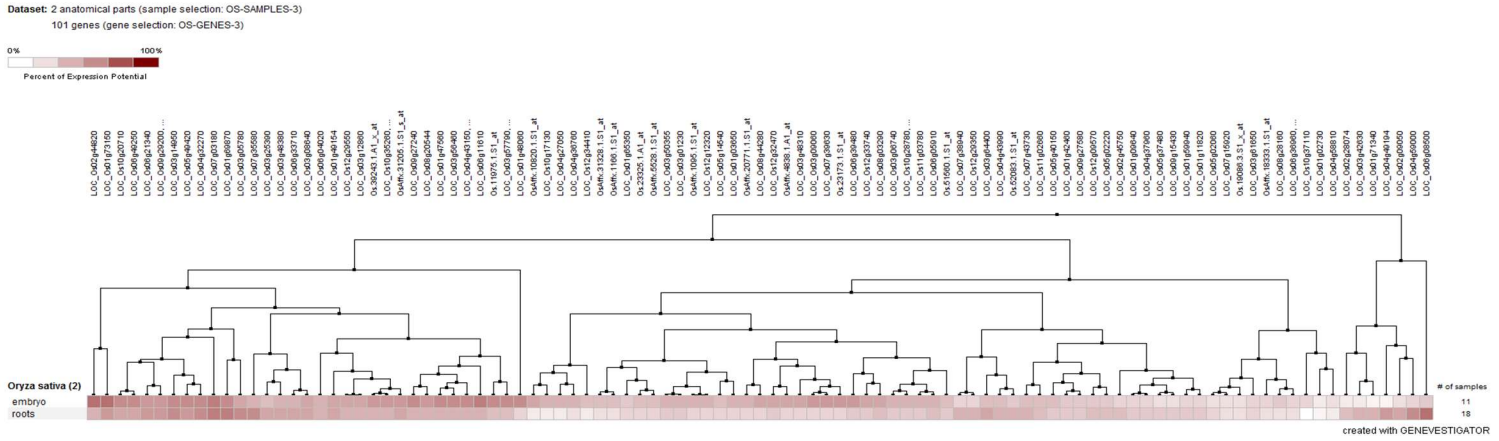

Figure 2.8: Hierarchical Clustering result for up-regulated genes generated using GeneVestigator

Appendix 2:

Hydroponic rice growth experiment plants parameter measurement result:

Box number	Variety	Growth condition	Shoot length (cm)	Root length-longest (cm)	Second root length (cm)	Root dry weight (g)	Shoot dry weight (g)
LP 1-1	*WT*	LP	71	84.6	71.5	1.326	2.99
LP 1-2	*RHL1-ov*	LP	75.4	66.1	66.1	1.407	2.476
LP 1-3	*RHL1-ov*	LP	78.7	69.7	69.4	1.366	2.507
LP 1-4	*rhl1*	LP	84.1	69.3	68.7	1.702	3.796
LP 1-5	*rhl1*	LP	71.6	59.6	58.3	0.453	1.058
LP 1-6	*WT*	LP	80.8	70.4	65.4	1.066	2.223
LP 2-1	*RHL1-ov*	LP	80.1	81.9	80.9	2.824	4.32
LP 2-2	*rhl1*	LP	78.9	57.4	55.4	1.142	1.942
LP 2-3	*rhl1*	LP	64.4	60.2	56.5	0.229	0.51
LP 2-4	*WT*	LP	82.3	67.6	66.4	1.663	3.121
LP 2-5	*WT*	LP	67.8	55.4	53.4	0.838	2.028
LP 2-6	*RHL1-ov*	LP	84.3	78.2	78.1	2.473	3.874
LP 3-1	*rhl1*	LP	75.5	79.6	77.4	1.563	2.62
LP 3-2	*WT*	LP	70.6	83.4	79.1	1.518	2.158
LP 3-3	*WT*	LP	72.4	83.7	83.1	1.655	2.797
LP 3-4	*RHL1-ov*	LP	80.4	84.5	83.5	1.596	2.583
LP 3-5	*RHL1-ov*	LP	83.3	79.8	78.9	2.292	4.111
LP 3-6	*rhl1*	LP	80.2	74.8	73.7	1.058	2.031
HP 1-1	*WT*	HP	106.5	60.5	59.4	1.478	7.028
HP 1-2	*RHL1-ov*	HP	94.2	59.3	59	1.811	8.201
HP 1-3	*RHL1-ov*	HP	103	67.1	61	1.743	8.971
HP 1-4	*rhl1*	HP	105.6	53.8	51.9	2.828	13.118
HP 1-5	*rhl1*	HP	98.7	57.2	56.4	2.041	9.288
HP 1-6	*WT*	HP	100.1	60.2	60.2	5.544	24.407
HP 2-1	*RHL1-ov*	HP	98.9	73.1	62.5	3.852	15.227
HP 2-2	*rhl1*	HP	94.8	52.4	52.1	2.674	9.777
HP 2-3	*rhl1*	HP	87.1	54.2	48.4	1.292	4.989
HP 2-4	*WT*	HP	93.4	56.1	55.6	1.374	10.376
HP 2-5	*WT*	HP	92.5	62	61.5	2.754	13.796
HP 2-6	*RHL1-ov*	HP	92.1	55	49.8	2.914	11.493
HP 3-1	*rhl1*	HP	102.2	71.6	62.2	4.654	18.078
HP 3-2	*WT*	HP	69.2	70.2	70.2	1.719	5.626
HP 3-3	*WT*	HP	103.1	60.6	60.4	3.896	16.105
HP 3-4	*RHL1-ov*	HP	92.1	71.5	61.4	3.338	11.139
HP 3-5	*RHL1-ov*	HP	89.2	70.1	68.3	3.313	12.948
HP 3-6	*rhl1*	HP	88.2	59.6	57.8	1.982	7.147

Appendix 3:

Phosphate content analysis made by Lancrop Labortories

Growth condition	Sample name	Sample Reference	Phosphorus (%)
HP	*WT*	OCT12 SHOOT P112 HP1-1	0.84
HP	*WT*	OCT12 SHOOT P112 HP1-6	0.62
HP	*WT*	OCT12 SHOOT P112 HP2-4	0.44
HP	*WT*	OCT12 SHOOT P112 HP2-5	0.58
HP	*WT*	OCT12 SHOOT P112 HP3-2	0.68
HP	*WT*	OCT12 SHOOT P112 HP3-3	0.73
LP	*WT*	OCT12 SHOOT P116 LP1-1	0.07
LP	*WT*	OCT12 SHOOT P116 LP1-6	0.09
LP	*WT*	OCT12 SHOOT P116 LP2-4	0.07
LP	*WT*	OCT12 SHOOT P116 LP2-5	0.06
LP	*WT*	OCT12 SHOOT P116 LP3-2	0.07
LP	*WT*	OCT12 SHOOT P116 LP3-3	0.06
HP	*rhl1*	OCT12 SHOOT P112 HP1-4	0.77
HP	*rhl1*	OCT12 SHOOT P112 HP1-5	0.75
HP	*rhl1*	OCT12 SHOOT P112 HP2-2	0.8
HP	*rhl1*	OCT12 SHOOT P112 HP2-3	0.74
HP	*rhl1*	OCT12 SHOOT P112 HP3-1	0.74
HP	*rhl1*	OCT12 SHOOT P112 HP3-6	0.99
LP	*rhl1*	OCT12 SHOOT P116 LP1-4	0.08
LP	*rhl1*	OCT12 SHOOT P116 LP1-5	0.08
LP	*rhl1*	OCT12 SHOOT P116 LP2-2	0.07
LP	*rhl1*	OCT12 SHOOT P116 LP2-3	0.07
LP	*rhl1*	OCT12 SHOOT P116 LP3-1	0.07
LP	*rhl1*	OCT12 SHOOT P116 LP3-6	0.09
HP	*RHL1-ov*	OCT12 SHOOT P112 HP1-2	1.03
HP	*RHL1-ov*	OCT12 SHOOT P112 HP1-3	0.94
HP	*RHL1-ov*	OCT12 SHOOT P112 HP2-1	0.89
HP	*RHL1-ov*	OCT12 SHOOT P112 HP2-6	1.03
HP	*RHL1-ov*	OCT12 SHOOT P112 HP3-4	0.97
HP	*RHL1-ov*	OCT12 SHOOT P112 HP3-5	0.79
LP	*RHL1-ov*	OCT12 SHOOT P116 LP1-2	0.08
LP	*RHL1-ov*	OCT12 SHOOT P116 LP1-3	0.08
LP	*RHL1-ov*	OCT12 SHOOT P116 LP2-1	0.07
LP	*RHL1-ov*	OCT12 SHOOT P116 LP2-6	0.08
LP	*RHL1-ov*	OCT12 SHOOT P112 LP3-4	0.14
LP	*RHL1-ov*	OCT12 SHOOT P116 LP3-5	0.07

Appendix 4:

Nutrient content of roots using ICP-MS 1:

Sample ID	Variaty	Weight	Digest volume	Na	Mg	P	S	K	Ca	B	Mn	Cu	Zn	Mo	Fe
		(g)	(mL)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)
LP1-1-1	*WT*	0.12	15.00	960.80	1876.20	726.72	6678.12	23540.81	1109.90	8.86	80.06	56.59	58.07	46.89	112.97
LP1-1-2	*WT*	0.20	15.00	1189.15	2129.80	876.70	7903.11	30184.43	1331.05	11.85	74.92	53.34	61.82	52.83	126.41
LP1-6-1	*WT*	0.10	15.00	810.37	1566.85	580.67	6493.85	18561.01	1432.29	9.88	25.84	67.76	40.42	85.99	131.44
LP1-6-2	*WT*	0.19	15.00	769.40	1758.17	586.74	7462.26	21667.41	1476.45	9.33	26.71	69.07	43.96	93.32	113.47
LP2-4-1	*WT*	0.11	15.00	644.66	1495.93	660.70	6736.89	21531.69	1231.25	10.03	16.38	39.67	67.38	68.02	155.30
LP2-4-2	*WT*	0.19	15.00	642.24	1722.18	677.28	8145.33	25234.20	1217.33	11.77	18.13	45.37	70.96	79.75	111.91
LP2-5-1	*WT*	0.11	15.00	1016.88	1512.57	572.97	6255.41	19349.81	1620.54	9.23	41.05	29.96	49.67	69.54	136.40
LP3-2-1	*WT*	0.10	15.00	687.19	1462.03	600.01	6714.83	23864.36	1111.83	10.18	14.96	32.81	44.27	46.39	103.52
LP3-2-2	*WT*	0.20	15.00	743.51	1584.89	571.22	7300.82	25391.73	1145.89	9.48	14.52	33.88	45.43	49.07	101.40
LP3-3-1	*WT*	0.10	15.00	924.45	1935.42	810.79	8171.45	27526.62	1746.99	12.85	42.65	37.23	67.58	46.54	158.95
LP3-3-2	*WT*	0.20	15.00	953.10	2004.64	782.19	8646.32	28015.75	1580.66	12.55	49.63	39.80	68.08	47.90	139.69
LP1-4-1	*rhl1*	0.11	15.00	628.83	1659.94	732.90	7106.83	22125.21	1102.51	10.34	13.86	49.57	45.34	79.10	88.35
LP1-4-2	*rhl1*	0.18	15.00	694.46	1994.78	788.36	8063.02	25725.14	1236.96	10.17	15.50	54.49	53.60	87.31	125.36
LP1-5-1	*rhl1*	0.11	15.00	860.13	1910.63	564.20	6789.04	15909.26	1805.12	9.08	54.62	93.69	50.12	93.07	147.38
LP1-5-2	*rhl1*	0.19	15.00	929.31	2235.03	638.31	7864.20	18636.58	1990.97	10.22	61.10	105.91	56.93	107.26	170.79
LP2-2-1	*rhl1*	0.11	15.00	603.18	1607.96	748.32	6978.79	23868.81	1138.14	8.96	14.13	35.33	42.15	59.19	105.76
LP2-2-2	*rhl1*	0.19	15.00	590.49	1609.74	682.74	7217.90	24398.13	1054.09	7.37	12.62	29.77	40.40	53.17	86.15
LP3-1-1	*rhl1*	0.10	15.00	732.03	1611.93	687.21	7127.16	24699.99	1139.86	12.51	15.93	22.14	48.51	38.34	91.51
LP3-1-2	*rhl1*	0.19	15.00	790.14	1751.38	707.93	7935.75	26353.16	1198.01	13.16	17.79	24.70	50.48	42.27	98.01
LP3-6-1	*rhl1*	0.10	15.00	805.50	1906.58	601.92	7298.89	20607.82	1399.37	12.29	19.19	40.62	50.97	60.74	172.62
LP3-6-2	*rhl1*	0.17	15.00	735.46	1793.93	558.26	7502.21	20907.38	1314.58	10.52	18.02	39.77	46.55	61.92	114.29
LP1-2-1	*RHL1-ov*	0.11	15.00	676.81	1826.41	856.80	7747.67	26412.44	1445.22	10.70	21.43	41.99	60.88	80.94	138.70
LP1-2-2	*RHL1-ov*	0.20	15.00	712.76	1887.24	859.87	8346.56	28076.90	1502.81	12.10	21.51	40.46	61.44	81.09	120.72
LP1-3-1	*RHL1-ov*	0.11	15.00	600.19	1556.41	683.59	7146.89	22878.64	1245.87	10.28	19.55	45.38	54.15	79.94	99.91
LP1-3-2	*RHL1-ov*	0.21	15.00	666.67	1734.02	691.27	7895.79	25589.31	1343.98	11.08	20.36	46.52	56.29	86.30	108.70

Nutrient content of roots using ICP-MS 2:

Sample ID	*Variaty*	Weight	Digest volume	Na	Mg	P	S	K	Ca	B	Mn	Cu	Zn	Mo	Fe
		(g)	(mL)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)
LP2-1-1	*RHL1-ov*	0.11	15.00	639.60	1474.55	608.22	6645.12	23934.10	1153.58	8.63	23.47	33.62	55.28	55.44	124.87
LP2-1-2	*RHL1-ov*	0.19	15.00	642.37	1647.59	622.02	7605.08	27349.68	1225.04	9.06	22.75	34.39	57.86	57.38	104.36
LP2-6-1	*RHL1-ov*	0.11	15.00	811.53	1631.49	784.02	7948.06	28827.61	1369.86	15.78	11.57	28.23	72.04	51.36	106.69
LP2-6-2	*RHL1-ov*	0.21	15.00	882.14	1795.64	904.46	8210.89	29658.06	1630.73	15.00	13.01	31.60	78.18	58.39	131.51
LP3-5-1	*RHL1-ov*	0.11	15.00	603.76	1377.02	627.25	6336.88	20113.37	984.09	7.38	12.51	32.96	65.53	51.94	99.71
LP3-5-2	*RHL1-ov*	0.20	15.00	580.99	1426.50	621.64	6709.18	21595.28	965.90	7.54	13.14	32.30	65.54	52.03	102.65
HP1-1-1	*WT*	0.11	15.00	8912.35	3058.28	3295.52	9867.21	13533.92	1785.02	7.82	36.76	69.01	62.53	24.18	422.00
HP1-1-2	*WT*	0.19	15.00	9807.27	3036.05	3050.94	10855.84	14430.87	1782.70	8.31	33.63	61.78	61.00	23.04	312.76
HP1-6-1	*WT*	0.11	15.00	8298.77	2368.03	2404.50	7063.79	8981.72	1418.55	3.55	12.99	96.51	48.96	17.68	270.33
HP1-6-2	*WT*	0.20	15.00	8690.07	2538.94	2615.33	7688.91	9501.14	1530.13	4.67	13.97	105.66	51.61	19.34	270.76
HP2-4-1	*WT*	0.11	15.00	8083.69	2251.24	2707.14	7572.52	9330.89	1596.55	4.15	23.20	76.77	43.30	14.57	500.44
HP2-4-2	*WT*	0.17	15.00	8238.82	2364.95	2835.78	7681.35	9646.06	1628.36	5.52	22.28	79.16	43.51	14.96	513.25
HP2-5-1	*WT*	0.12	15.00	6996.24	2162.40	1862.63	6225.12	6072.26	1469.01	5.55	15.25	60.39	39.52	22.13	412.34
HP2-5-2	*WT*	0.19	15.00	7128.58	2318.85	2048.40	6367.20	6591.83	1547.31	7.63	16.23	62.63	41.29	22.96	428.60
HP3-2-1	*WT*	0.08	15.00	7561.24	2998.45	3819.92	8994.05	14658.36	1987.39	6.17	29.28	116.79	63.92	15.41	851.41
HP3-2-2	*WT*	0.18	15.00	4626.63	1914.33	2199.58	5530.69	8786.74	1152.75	3.38	15.79	67.42	39.48	8.75	514.55
HP3-3-1	*WT*	0.13	15.00	10236.09	2908.80	2942.07	11064.38	16686.25	1928.95	6.71	19.28	54.76	76.13	10.84	611.19
HP3-3-2	*WT*	0.19	15.00	10187.72	3085.39	3615.49	11416.72	18040.18	2155.70	9.46	21.90	64.39	85.30	12.73	639.85
HP1-4-1	*rhl1*	0.13	15.00	10296.60	2866.02	3437.27	11183.37	13449.89	1548.79	5.02	30.99	74.62	46.14	18.04	293.93
HP1-4-2	*rhl1*	0.18	15.00	10437.33	2942.67	3775.61	11569.06	13978.99	1688.90	6.06	33.88	82.95	46.76	19.59	279.32
HP1-5-1	*rhl1*	0.10	15.00	9047.41	3062.29	3462.84	10044.92	13213.93	1629.60	7.01	31.64	66.91	55.75	18.63	291.05
HP1-5-2	*rhl1*	0.20	15.00	9651.66	3248.44	3475.29	10829.45	13940.82	1680.49	8.10	30.84	63.38	56.28	18.60	297.77
HP2-2-1	*rhl1*	0.12	15.00	7641.09	2366.33	3336.71	8284.86	11037.61	1581.49	4.09	16.01	91.71	48.80	11.91	767.03
HP2-2-2	*rhl1*	0.17	15.00	7691.21	2398.80	3639.67	8512.11	11344.69	1738.26	4.12	17.58	104.66	53.11	13.39	764.34
HP2-3-1	*rhl1*	0.12	15.00	5833.10	2764.14	2921.31	6915.89	12983.46	2308.98	7.15	36.89	73.15	51.62	17.30	309.21
HP2-3-2	*rhl1*	0.21	15.00	6348.70	3068.72	3236.78	7786.78	13296.00	2728.11	7.23	41.54	78.78	53.08	19.46	364.34
HP3-1-1	*rhl1*	0.10	15.00	8183.08	3237.04	3650.75	10607.21	20501.55	1577.96	5.05	23.83	58.66	52.03	9.29	395.43
HP3-1-2	*rhl1*	0.19	15.00	8385.32	3146.19	3318.52	11200.05	20483.05	1480.20	5.72	21.44	48.37	49.93	8.40	371.99

Nutrient content of roots using ICP-MS 3:

Sample ID	*Variaty*	Weight	Digest volume	Na	Mg	P	S	K	Ca	B	Mn	Cu	Zn	Mo	Fe
		(g)	(mL)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)
HP3-6-1	*rhl1*	0.11	15.00	8940.16	2899.33	3247.71	9499.51	12505.03	2435.22	8.29	29.64	96.80	90.28	16.77	1060.43
HP3-6-2	*rhl1*	0.18	15.00	9796.98	3159.46	2833.40	10407.32	13045.62	2307.55	7.03	26.47	82.70	85.49	14.87	1022.21
HP1-2-1	*RHL1-ov*	0.11	15.00	9569.43	3072.44	3439.58	11138.16	14309.19	1729.06	6.88	39.43	95.97	74.93	25.25	280.72
HP1-2-2	*RHL1-ov*	0.18	15.00	9675.91	3079.72	3415.40	10928.20	14265.84	1753.31	7.50	39.67	95.49	68.49	25.94	275.55
HP1-3-1	*RHL1-ov*	0.13	15.00	8682.25	2798.30	3380.69	9733.12	13738.00	1832.32	7.11	25.25	82.22	60.48	24.82	277.97
HP1-3-2	*RHL1-ov*	0.19	15.00	9234.39	3146.72	3194.67	10501.12	14494.66	1846.98	6.78	25.66	82.18	60.40	25.23	397.54
HP2-1-1	*RHL1-ov*	0.12	15.00	7177.08	1946.72	2599.03	6513.21	8585.40	1652.74	4.26	12.66	108.72	47.64	14.12	515.17
HP2-1-2	*RHL1-ov*	0.19	15.00	8049.18	2214.60	2871.42	7333.73	9587.38	1848.29	5.41	14.29	127.93	54.69	15.39	535.59
HP2-6-1	*RHL1-ov*	0.11	15.00	8021.69	2211.67	2656.31	7253.97	8230.05	1906.74	5.30	15.07	116.55	55.48	18.98	677.47
HP2-6-2	*RHL1-ov*	0.17	15.00	8865.89	2554.84	3035.96	8013.08	9146.30	2134.01	8.38	17.15	124.60	88.74	20.63	775.74
HP3-4-1	*RHL1-ov*	0.13	15.00	9130.88	2999.17	2938.33	9853.45	12475.05	2339.47	10.94	25.73	98.46	73.69	16.18	1015.18
HP3-4-2	*RHL1-ov*	0.18	15.00	9032.82	2801.11	2701.53	9869.71	12055.41	2125.56	10.07	23.04	87.48	66.26	14.53	900.06
HP3-5-1	*RHL1-ov*	0.14	15.00	7077.22	2824.52	2860.49	8370.72	12019.40	1890.79	9.00	21.11	60.51	134.90	8.69	563.87
HP3-5-2	*RHL1-ov*	0.19	15.00	7024.36	2836.23	3013.18	8561.78	12379.13	1904.29	7.82	20.73	60.35	145.58	8.89	547.76