Molecular Evidence of Neo-tetraploids in *Arabidopsis thaliana* leaf elevated potassium.

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Abstract

Crop species that are polyploid are more likely to exhibit unique phenotypes, such as increased organ size and strong resistance to a variety of abiotic stressors that are relevant to the climate. In the model species *Arabidopsis thaliana* neotetraploids show elevated level in potassium (K+) in leaves. It was noted there is an alteration in well-known components of the K+ homeostasis. Mutational analysis revealed that AKT1, HAK5 and CIPK23 are not responsible for the observed increase. Instead, signalling of abscisic acid might connect with leaf elevated potassium. This review gives a brief outline about whole genome duplication and role of K+ in plants and their distribution. Also, it discusses ABA synthesis, response to neo-tetraploids.

Keywords; *Arabidopsis thaliana*, Neo-tetraploids, HAK5, AKT1 and K+ elevation.

1. Introduction

Whole- genome duplication or WGD is doubling the genome that provides raw material and elevate genomic complexity. WGD can help in phenotypic evolution and the ensuing alterations in the future of replica genes, as has been steadily discovered in recent times (Moriyama Y et al., 2018). On the other hand, polyploidy is common and many diploids have polyploid heritage can trace via sequencing. The emergence of extensive and varied taxonomic lineages can be linked to a portion of these ancient polyploidy events (Carretero-Paulet, L et al., 2020). Furthermore, the majority of crops are neopolyploids, indicating a significant role for WGDs in phenotypic variation, speciation, and domestication (Van de Peer et al., 2017). In addition to offering a wealth of material for mutants, evolutionary adaptation, and selection, polyploidy in plants has emerged as a key strategy for advancing both evolution and plant breeding. WGD has been connected to both speciation and habitat adaptability. Polyploids, unlike their diploid parents, often live in larger or different environments. There is a tendency for polyploidy to increase during periods of climate change, and polyploid incidence to rise with latitude globally (Parisod C et al., 2010) (Bomblies, 2020). Due to its associations with advantageous characteristics like increased resistance to stress, bigger seeds or fruits, or altered metabolite content, polyploidy is also of great interest to the agricultural

community. These patterns provide credence to the notion that polyploidy is frequently connected to innovation (Rice A et al., 2019). Neo-tetraploids were consequently thought to survive for a brief period of time during periods of environmental change that would negatively impact their diploid progenitors, undermine the evolutionary benefit of their local adaptation (Anatskaya, O. V., and Vinogradov A. E. 2022).

Additionally, according to previous research, whole genome duplication may have benefits for short-term survival, as evidenced by the increased resistance to salinity and drought shown by neo-tetraploid Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*), and citrange (*Citrus sinensis* L. Osb. × *Poncirus trifoliata* L. Raf.) (Yang et al., 2014) (Chao et al., 2013) (Pozo et al., 2014) (Ruiz et al., 2016). One of the first model organisms in history, *Arabidopsis thaliana* was used in a number of the most extensive studies to improve organelle, tissue, cell, or sub-cellular proteome profiling, genome annotation, developmental processes, and responses to biotic and abiotic challenges using differential relative and absolute quantitative strategies (Wang et al., 2021). Additionally, neo-tetraploid rice and Arabidopsis have increased jasmonic acid (JA) and abscisic acid (ABA) signaling, respectively, which is again in line with possessing inherent resistance to abiotic stress tolerance caused by whole-genome duplication.

Based on these data, it appears that duplicating a whole-genome can increase fitness effect even in the presence of unfavorable environmental circumstances. Polyploids are known to have better resistance to abiotic stressors, although the precise molecular mechanisms causing these adaptations are yet unknown (Fox et al., 2020).

A very useful platform for examining the molecular features of variance in plants is provided by transcriptome data derived from RNA-seq. Transcriptome analysis in rice, maize, and wheat has demonstrated the intricacy of gene expression patterns linked to diversity and differential gene expressions in various tissues (Park SY et al., 2022).

This review will briefly outline the transcriptomic response of neotetraploid *Arabidopsis* which has elevated levels of leaf potassium and their transcriptomic changes to ABA signaling. The review will show, we lack knowledge of concrete genes involved in the molecular basis for observed phenotypes. This can be overcome by comparing the gene expression in neotetraploid with wild type with those mutants of candidate genes.

2. Neo-tetraploids and Whole Genome Duplication

An expansion in our knowledge of plant structure and function has resulted from the performing of today's science, the development of analytical technologies, and the use of *Arabidopsis thaliana* as a model organism. Research study on *Arabidopsis* has added for the advancement of modern biology. The quantity of genomic copies found in a single nucleus is called ploidy. A diploid cell's nucleus has two copies of its genome; if the cell and nucleus have more than two copies (such as four, or eight), they are called polyploidy. Tetraploid refers to four copies of genome in a single nucleus (Woodward AW and Bartel B 2018). Whole-genome duplication (WGD) has been a common process in plant evolution, and evolved polyploidy plants have demonstrated more genetic variety, which may offer a means of increasing genetic diversity and cultivating exceptional cultivars (Bomblies, K. 2020). By duplicating the whole genome, a diploid becomes tetraploids. Neo-tetraploids of Arabidopsis show evidence of elevation of leaf potassium when compared to its diploid parent (Chao et al., 2013) (Yu et al., 2009). An increase in cell size is one of WGD's most reliable effects. Almost every cellular function might be impacted by a modification in something so basic. A system may tremble in response to certain impacts of cell size change, but others may be more seismic and call for an evolutionary reaction. The ploidy shifts-related change is the reason for cell sizes has significant and varied consequences regarding the biology of organisms and cells. (Bomblies, K. 2020).

"Circumstances within the nucleus that influence the phenotype without regard to the information contained in the DNA" were what Bennett referred to as "nucleotypes" in 1971. It has also been shown that the WGD-related alterations in cell size may have significant evolutionary ramifications. Levin noted that WGD's effects on biochemistry, physiology, and development can "transport a population to a new area of adaptation." (Bennett MD 1971). Auto- and allo-polyploids are the two categories of polyploids. Allo-polyploids start followed hybridization episodes or from the fusion of unreduced gametes from genetically distinct parents. Autopolyploids emerge within-species WGD. The early impacts of WGD are now well covered in the literature, and several studies have been done to examine the nearest diploid relatives of well-established polyploids. (Robinson et al., 2018).

While studies of established tetraploids allow us to determine whether observed differences resulted from whole genome duplication or from later "conventional" adaptive evolution, studies of neo-polyploids allow us to identify the immediate consequences of WGD without being aware of their final evolutionary destiny (Bomblies K et al., 2014). These studies have advantages and disadvantages. As has been said, we may obtain a more comprehensive understanding of the advantages and disadvantages of polyploidy, as well as potential cellular and developmental adaptations, by contrasting diploids with both recently formed autotetraploids and developed autotetraploids (Hegarty et al. 2013). Of the 67 species surveyed, 46 are neopolyploids, meaning they were naturally autotetraploids. It was therefore obvious that numerous entire genome duplications result in similar symptoms. Cell size growth, especially in the case of stomatal guard cells, is one of the most commonly documented features. This is not unexpected, considering that DNA amount and ploidy have long been recognised to correlate with cell size.

The degree to which this is true varies, however, both within and across species, and particularly between different cell types within an organism (Ario DM et al., 2019). Growing stomata to facilitate greater gas exchange is one way to boost photosynthetic rates, and certain polyploid do in fact exhibit higher gas exchange rates (Franks PJ et al., 2007). The response of the photosynthetic rate per leaf area to whole genome duplication varies, probably reflecting differences in leaf morphology. In contrast to polyploidy, the photosynthetic rate per chloroplast stays constant, while for every cell size, there is a steady increase in the number of chloroplasts and photosynthetic rate (Drake et al ., 2013). Stress tolerance is a characteristic of polyploid that is frequently described. Greater drought tolerance is among the most dependable tolerances for established and neo-polyploid. Numerous neopolyploids have also been discovered to have improved salt tolerance (Beest M et al., 2012). Other stress resistances are more erratic; polyploids exhibit varying degrees of resistance to heat and cold, as well as enhanced resistance to UV, low freezing tolerance and low hydric tolerance (te Beest et., 2012). The rate of water transpiration and the rate at which CO2 is absorbed by the plant and thus the rate of photosynthetic activity are both influenced by the size and density of stomata. This is shown, for example, in Arabidopsis thaliana, where plants

exhibit reduced gas exchange in proportion to their stomatal densities and photosynthetic rates, whereas mutants with larger densities have higher levels of CO2 assimilation in high light. For polyploids it is un-clear what to expect because they have fewer stomata and bigger stomata (Bussis et al., 2006). Data on gas exchange at the leaf level lend credence to the mechanistic interpretation of carbon and water fluxes in plants. These fluxes are essential to understanding how ecosystems work, restrict the parameterization of models of the terrestrial biosphere, help us understand how plants respond to environmental change on a global scale, and are crucial to attempts to increase agricultural productivity (Ely KS et al., 2021).

Mutational research showed that the variation in leaf K resulting from whole-genome duplication is not dependent on the K+-uptake transporters K+ Transporter 1 (AKT1) and High affinity K+ transporter 5 (HAK5). However, the greater concentration of leaf K in neo-tetraploids required signals linked to abscisic acid (Fischer et al., 2022). A second method of ploidy modification, endoreduplication, takes place during differentiation in individual cells. It allows cells to become much more ploid than the organism's "base" ploidy level. Endoreduplication takes place as an alternative to the mitotic cell cycle. In normal cycle—which includes the gap (G) and DNA synthesis (S) phases and omits the mitotic (M) phase—maintains multiple copies of the genome in a single nucleus. As cells divide into two 2C (diploid) daughter cells during the mitotic cycle, endoreduplicate cells remain at 4C and can undergo subsequent endocycles to become 8C, 16C, 32C, and so on (Robinson et al., 2018).

Endoreduplication doubles the number of chromatids on each chromosome (C); if chromosomes stay polytene, it will affect N, the overall number of chromosomes, as well. Although whole genome modification and endoreduplication both produce polyploid cells, their cytologies are most likely not the same. Whole genome modified polyploid cells need to go through mitotic divisions with additional copies of their genome due to fact that WGM makes every zygote and in all descendant somatic cells more ploidy. On the other hand, endoreduplicated cells are developing during terminal differentiation (Katagiri et al., 2016). Some endoreduplicated chromosomes have been observed in plants using microscopy; however, trichomes, xylem cells, and suspensor and tapetum cells have all been reported to exhibit complete or partial polyteny. It has

been discovered that endoreduplicated plant chromosomes are partly polytene or incompletely aligned, and as ploidy increases, chromatid synapsis tends to become less pronounced (Roeder et al., 2010).

3. K uptake and distribution

Potassium (K+), being among the most significant mineral nutrition components, is involved in several physiological processes in plants and impacts crop quality and yield (Gebreslassie HB 2016). Their cellular levels are 100 mM in the cytosol of plant cells.

K+ is absorbed by the plant root via the cortical and epidermal cells. After entering the stele, it travels to the shoot and is then dispersed to the leaves. K+ has an impact on several functions within plants, including growth, the ability of the plant to withstand biotic and abiotic stress, and organ movement (Ragel et al., 2019). Genes belonging to the KT/HAK/KUP family are frequently linked to the transport of K+ across membranes and the supply of K+. K+ carriers in this class are encoded by single-copy genes called *kup* in bacterial genomes. This family's members have been widely linked to high-affinity K+ uptake from the soil, but other members might play roles in low-affinity, high-affinity, or both types of transport. These roles may include K+ translocation, plant water movement control, resistance to salt, transport of other alkali cations, osmotic/drought responses, and plant developmental processes like auxin distribution and root hair growth (Wang Y et al., 2013).

The plants have developed complex systems for the absorption, efflux, and distribution of K+ inside cells and across organs. These activities take place at the level of individual cells, organs, and entire plants. According to how they react to the membrane potential, plant voltage-gated K+ channels are categorized into three subfamilies: Inward-rectifying (Kin) channels: AKT1, AKT6, KAT1, and KAT2 in Arabidopsis; they open at hyperpolarized membrane potentials to permit K+ absorption. Outward-rectifying (Kout) channels, which include the Stelar K+ outward rectifier channel SKOR and gated outwardly rectifying K+ channel GORK channels, facilitate K+ release because they open at depolarized membrane potentials. Weakly rectifying (Kweak) channels are represented by AKT2, which is capable of mediating both K+ uptake and release (Yu et al., 2020). When external K+ concentrations increase, the absorption of K+ by roots displays biphasic kinetics. This is indicative of high- and low-affinity transport systems,

which function at low (<1 mM) and high (>1 mM) external K+ concentrations, respectively. K+ mostly enters the membrane through channels when it is present in the soil solution at high concentrations (Ragel et al., 2019).

The electrochemical gradient's channel permits ion movement. When the concentration of K+ is low, the cell must use active transporter systems to pull K+ against the electrochemical gradient. Research on a variety of plant species has shown that the membrane may be involved in K+ absorption in the high-affinity range of K+ concentrations because it is sufficiently hyperpolarized and considerably electronegative within the channels (Santa-Maria et al., 2018). Specifically HAK5 is involved in K+ uptake at low external concentrations. Along with HAK5 transporters, Akt1 channels are also plays it part in the main systems for K+ uptake in plants. On the other hand, depending on the kind of plant and the ionic external concentration of transported substrates (primarily K+, NH4 +, and Na+), the NH4+ sensitive and insensitive routes seem to contribute differentially to high-affinity K+ absorption (Spalding et al., 1999). Research studies point out those transporters KT/HAK/KUP mediates NH4+ sensitive component of K+ uptake (HAK1-like transporters), whereas inward-rectifier K+ channels (AKT1-like channels) constitute the NH4 + -insensitive pathway (Santa-Maria et al., 2000). Research findings indicated that the inward-rectifying K+ channel AKT1 is activated by Calcineurin B like interacting protein kinase23 (CIPK23/CBL1-9) complexes. Therefore, the K+ transporter HAK5, which is in charge of high-affinity K+ absorption in roots, is upregulated by Calcineurin B like interacting protein kinase23 (CIPK23/CBL) modules. As K+ nutrients, these complexes are critical to the growth and development of plants. The Calcineurin B like interacting protein kinase23 also involves in the regulation of NO3- uptake. The Calcineurin B like interacting protein kinase23 (CIPK23/CBL1-9) activates AKT1 and the transcriptional gene HAK5 during the time of K+ scarcity (Straub et al., 2017). For the HAK5 transcriptional activity by CIPK23 complex depends upon the Ser/Thr kinase catalytic domain and the C- terminal domain of CIPK23. Perceiving and reacting to K+ by plants appears to be mediated by Ca2+, cell membrane potential, the hormones ethylene, reactive oxygen species (ROS), jasmonic acid or cytokinins and by K+ channels. K+ deprivation in Arabidopsis causes an upregulation of HAK5 transcription. The

hyperpolarization of the cell membrane results in the rapid accumulation of ROS. Ethylene mimicking components activate promoter AtHAK5. In addition to this, hormones also play a role as sensors, and transcription factors bind to the AtHAK5 promoter (Rubio et al., 2010).

4. **ABA responses and stress tolerance;**

Abiotic stress refers to the detrimental impact of non-living substances on living things within a certain ecosystems. Salt, drought, severe temperature and supplementary environmental conditions are among the stressors. Globally, drought and hypersalinity are the primary abiotic factors linked to crop failure (Wilkins et al., 2016). The Primary abiotic factor is not truly known but it might be restricted cellular water availability in Arabidopsis, and likely in other Embryophyta, that leads to the production of ABA and the subsequent activation of signalling processes. Elevated ABA concentrations maintain seed dormancy, decrease of water transpiration through stomatal pores and suppression of germination and the lateral root development (Hauser et al., 2011).

The plastidal 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway is used to synthesize the isoprenoid plant hormone abscisic acid (ABA). In contrast to the structurally similar sesquiterpenes, which originate from farnesyl diphosphate (FDP), a precursor generated from mevalonic acid, the cleavage of C40 carotenoid in MEP is where ABA's C15 backbone is formed. Zeaxanthin is the first committed ABA precursor. The proximal ABA precursor, xanthoxin, is then further oxidized to ABA via abscisic aldehyde. This process involves a series of enzyme-catalyzed reactions and isomerizations via violaxanthin and a final cleavage of the C40 carotenoid by a dioxygenation reaction (Zhang et al., 2020) (Nambara E et al., 2005).

The calcium dependent phosphorylation pathway may be the origin of the abiotic stress that triggers many ABA biosynthetic genes, including zeaxanthin oxidase (ZEP), 9-cis-epoxycarotenoid dioxygenase (NCED), ABA-aldehyde oxidase (AAO), and molybdenum cofactor sulfurase (MCSU). Abscisic acid (ABA) content varies depending on the rate at which the hormone is produced and broken down in a particular plant tissue. Therefore, to fully comprehend how this hormone controls plant growth and development, it is necessary to identify every gene implicated in metabolism (Cutler et al., 2010). Through the isolation of auxotrophic mutants, nearly all of the biosynthetic genes have been

found thus far. On the other hand, modern genomic techniques have shown that, among many other ABA catabolic pathways, the Arabidopsis CYP707A genes encode ABA 8'-hydroxylases, which catalyse the initial committed step in the main ABA catabolic pathway (Hauser et al., 2011).

The recently discovered pyrabactin resistance (PYR)/regulatory component of ABA receptor (RCAR) family of ABA receptor proteins, which function as negative regulators of members of the PROTEIN PHOSPHATASE 2C (PP2C) family, make up the core ABA signalling components (Fig.1). SNF1-RELATED KINASE 2 (SnRK2) protein kinases are activated when PP2C activity is inhibited. These kinases then target downstream components such as transcription factors, ion channels, and NADPH oxidases. These elements come together to generate an intricate network of ABA signalling (Finkelstein et al., 2008)

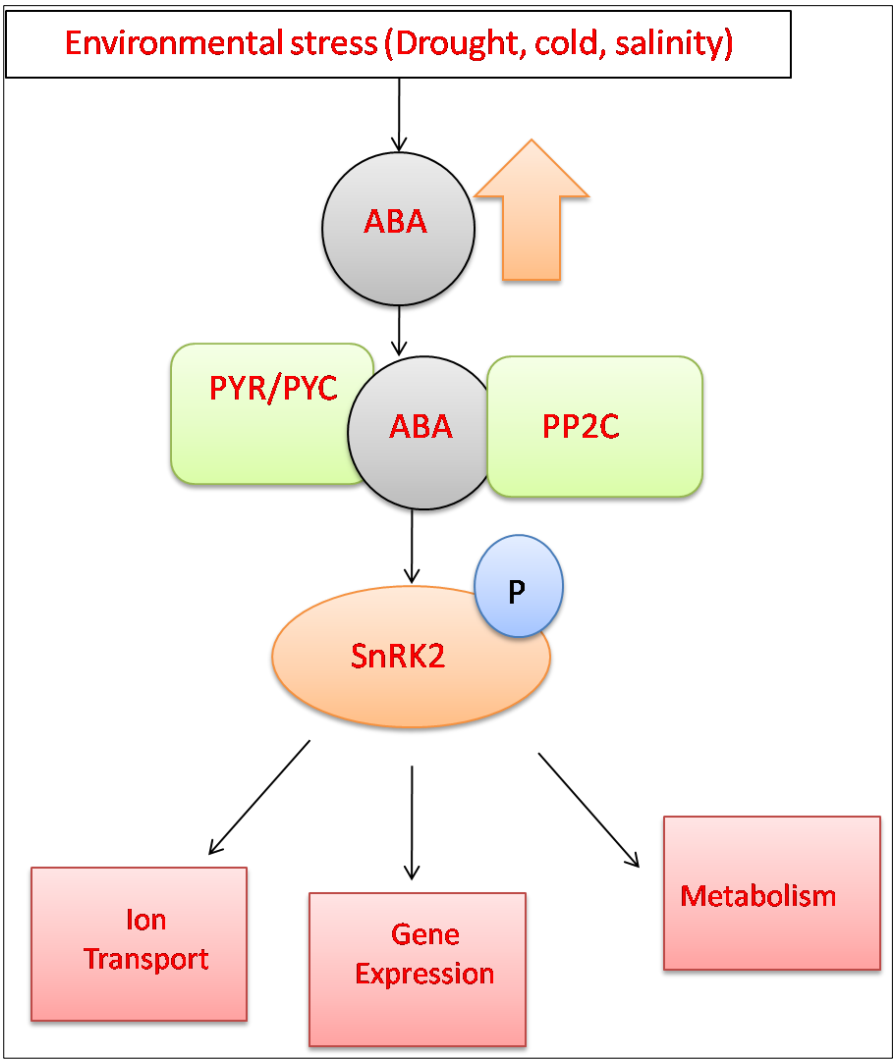

Figure. 1. Abscisic acid signalling compounds. {Abscisic acid (ABA), pyrabactin resistance (PYR), SNF1-RELATED KINASE 2 (SnRK2) protein kinases, P – phosphorous}.

The improved stomatal closure, salt tolerance, and drought resistance of neo-tetraploids Arabidopsis are compatible with their pre-activated ABA response, as shown by their positively regulated ABA signaling (Qian D et al., 2019) Guard cell migration, ABA signalling, and salinity tolerance are all related to K homeostasis.

Guard cell outward-rectifying K+, or GORK, is the voltage-gated channel that is the primary outward-rectifying K+ channel in guard cells of Arabidopsis, which aids in K+ efflux to reduce turgor and stomatal closure. Furthermore, GORK is thought to be a key route for stress-induced K+ leakage from root cells, such as when roots are exposed to high salt levels. This is because GORK origin in the outer cell layers of roots including

the cortex, root hairs, and epidermis (Getnet Dino Adem et al., 2020). Increasing amounts of abscisic acid (ABA) and jasmonate dramatically upregulate both the GORK channel activity and expression level. GORK transcripts were up-regulated in response to conditions that increased endogenous ABA concentrations, such as drought, osmotic stress, or cold (Shabala S. and Pottosin, I. 2014). The K+ supply and K+/Na+ balance must be maintained by high-affinity Stress conditions like K+ deficiency or salinity that directly affect K+ acquisition should cause transcriptionally or post-translational activated K+ absorption pathways. Abiotic stressors like as salt stress and maybe others cause changes in expression of AtHAK5 or response of low-K+ in addition to poor nutrition environments. AtHAK5 expression is not induced by mild salt stress, although it does gradually rise in response to elevated NaCl concentrations (Lara et al., 2020). This implies that excessive Na+ concentrations might be interpreted by plants as K+ shortage. Salt stress, inhibits decreased K+ levels which induce AtHAK5 expression in Arabidopsis.

As in our study, Neo-tetraploids Arabidopsis increase salinity tolerance and thereby maintaining Na+/K+ Homeostasis and increasing the abiotic stress tolerance but the knowledge in above mentioned responses are less and scientifically is not yet proven. The genotypes and phenotypes are not precise. There is no detailed description of post translational modifications of ABA core compounds and their relation to neo-tetraploids K+ elevations. To address this knowledge gap molecular studies need to carry down and so we can identifies the genes which are mainly takes place in the ABA stress tolerance responses. To identify the responsible genes and the phenotype characters – the wild type diploid and neo-tetraploids differentially expression genes will be compared. Those isolated genes are play as tools which can provide a result for the test hypothesis.

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"Molecular Evidence of Neo-tetraploids in *Arabidopsis thaliana* leaf elevated potassium."

A Project report

Submitted to the University of Nottingham, School of Biosciences, Sutton Bonnington LE12 5RD UK in partial fulfillment of the requirements for the award of the degree of

MRes Biomolecular Technology

Submitted by

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ABSTRACT

Polyploid crop species are prone to display distinctive traits, notably larger organs and robust tolerance to a range of abiotic stresses that are climate-relevant. Neotetraploids of the model species Arabidopsis thaliana have increased potassium (K+) levels in their leaves. Reputable elements of the K+ homeostasis were seen to be altered. It was found through mutational research that the observed increase is not caused by AKT1, HAK5, or CIPK23. Alternatively, abscisic acid signalling may be linked to increased potassium in leaves. Our goal was to determine common and ploidy-specific responses to a K uptake transporter deficiency by analyzing molecular alterations in diploid and neo-tetraploid wild type and K-transporter mutants. The eight homozygous mutant seeds (*Col-0* 2x, *Col-0* 4x, *akt1-2* 2x, *akt1-2* 4x, *hak5-3* 2x, *hak5-3* 4x, *akt1-2/hak5-3* 2x and *akt1-2/hak5-3* 4x) from *Arabidopsis thaliana* were grown in an artificial medium. Then, the root length were calculated and plotted as a graph by using RSTUDIO (Desktop Version: 2024.04.2+764). The RNA samples were extracted from the roots as well as the shoots. A Nanodrop spectrophotometer was utilized to quantify the amount of RNA present in the shoot. To assess the gene expressions, RNA was converted into complementary DNA and run through a polymerase chain reaction. The gene expressions were examined using agarose gel electrophoresis. In data analytics, the Venn diagram was made to compare several genotypes. PANTHER and ThaleMine were the programs used for gene ontology enrichment analysis and gene annotations. The separated genes serve as instruments that may offer a result for the hypothesis that is being investigated.

Keywords; *Arabidopsis thaliana*, Neo-tetraploids, HAK5, AKT1 and K+ elevation.

1. Introduction

The process of whole genome duplication (WGD) involves the doubling of the genome, resulting in the generation of more genetic material and increased genomic complexity. As has been gradually shown in recent years, WGD can aid in phenotypic development and the subsequent changes in the future of replica genes (Moriyama and Koshiba-Takeuchi, 2018). In the course of plant evolution, WGD has frequently occurred (Panchy et al., 2016). Polyploidy plants that have developed have shown more genetic variation, suggesting that developing superior cultivars and boosting genetic diversity may be possible (Bomblies, 2020). The term "tetraploid" describes a single nucleus with four copies of the genome. A diploid becomes a tetraploid by copying the whole genome. When compared to their diploid parent, neo-tetraploid Arabidopsis plants have elevated leaf potassium levels (Chao et al., 2013) (Yu et al., 2009). Furthermore, heightened jasmonic acid (JA) and abscisic acid (ABA) signalling have been seen in neo-tetraploid rice and Arabidopsis, respectively (Anderson et al., 2004) (Forcat et al., 2008). This is consistent with their innate ability to withstand abiotic stress (Saharan et al., 2022). While *AtHAK5* expression does progressively increase in response to increased NaCl concentrations, it is not caused by mild salt stress (Lara et al., 2020). This suggests that plants may perceive high Na+ concentrations as a K+ deficit (Assaha et al., 2017). Salt stress prevents lower K+ levels in Arabidopsis from causing an increasing in *AtHAK5* expression. A study including mutations revealed that the diversity in leaf K brought about by whole-genome duplication is independent of the K+-uptake transporters K+ Transporter 1 (AKT1) and High-affinity K+ transporter 5 (HAK5) (Fischer et al., 2022). According to (Gebreslassie, 2016), potassium (K+) is an important mineral nutrition component that affects crop quality and production and is involved in various physiological processes in plants. The supply and transport of K+ across membranes are commonly associated with genes in the KT/HAK/KUP family. Bacterial genomes contain single-copy genes termed *kup* that encode K+ carriers in this class (Tian and Imanian, 2023). Members of this family have been extensively associated with high-affinity K+ absorption from the soil (Wang and Wu, 2013). The

distribution, efflux, and absorption of K+ inside cells and among organs are handled by intricate mechanisms that plants have evolved. In Arabidopsis, the inward rectifying channels AKT1, AKT6, KAT1, and KAT2 can open to allow K+ absorption at hyperpolarised membrane potentials (Ragel et al., 2019). They open at depolarised membrane potentials, outward-rectifying (Kout) channels, such as the gated outwardly rectifying K+ channel GORK channels and the Stelar K+ outward rectifier channel SKOR, aid in K+ release. AKT2, which may mediate both K+ uptake and release, is a weakly rectifying (Kweak) channel (Yu et al., 2020). K+ absorption at low external doses is specifically mediated by HAK5. In addition to HAK5 transporters, Akt1 channels are involved in the primary pathways for K+ absorption in plants. Studies reveal that the NH4+ sensitive component of K+ uptake is mediated by the transporters KT/HAK/KUP (HAK1-like transporters), whereas the NH4 + insensitive route is made up of inward-rectifier K+ channels (AKT1-like channels) (Santa-María et al., 2000). It has been shown that polyploids have enhanced resistance to abiotic stressors, although the precise molecular processes causing these reactions are yet unknown (Van De Peer et al., 2021). Both the genetics and the phenotypes information are not clear. The relationship between neo-tetraploids' elevated K+ levels and the post-translational changes of ABA core components is not thoroughly explained. One way to get around this is to compare the gene expression of plants with gene mutations in important K uptake transporters in neotetraploids with diploids. Transcriptome data obtained from RNA-sequence provide a very helpful platform for investigating the molecular characteristics of variation in plants. The complexity of gene expression patterns connected to diversity and differential gene expressions in different tissues has been shown by transcriptome analysis in wheat, maize, and rice (Park et al., 2022). Both the shoot and the root RNA samples are extracted. An RNA concentration was measured using a Nanodrop spectrophotometer. RNA was transformed into complementary DNA and subjected to a polymerase chain reaction in order to evaluate the gene expressions. Agarose gel electrophoresis was used to see the gene expression. The Venn diagram was created in data analytics to compare several genotypes. Software for gene ontology and annotation was PANTHER and

ThaleMine. The separated genes function as tools that can yield an outcome for the hypothesis under examination. We investigated molecular changes in diploid and neo-tetraploid wild type and K-transporter mutants to identify common and ploidy specific responses to a lack of K uptake transporters.

Methods

1.1. Seed Sterilization.

The homozygous mutant seeds (*Col-0* 2x, *Col-0* 4x, *akt1-2* 2x, *akt1-2* 4x, *hak5-3* 2x, *hak5-3* 4x, *akt1-2/hak5-3* 2x and *akt1-2/hak5-3* 4x) were sterilized before being placed on growth culture media. The eight homozygous seed lines were placed in an Eppendorf tube. 500µl of 70% Ethanol were added to all the tubes and they were incubated for 15 minutes. After incubation, the supernatant liquid was removed. Then, the seeds were washed with 100% ethanol twice; the liquid removed part completely, and the seeds were allowed to dry. This procedure was performed in a laminar airflow chamber.

1.2. Growth assays for Arabidopsis an artificial medium.

Hoagland medium is the most used nutrient solution to measure the growth of *Arabidopsis thaliana* (van Delden et al., 2020). In our experiment, the media preparation, sowing and harvesting procedure were carried out for three times (triplicate). In a 1L measuring flask, 100µl of ammonium dihydrogen phosphate ($NH_4H_2PO_4$) and 200µl of magnesium sulfate ($MgSO_4$) were added. 280µl of calcium nitrate ($Ca(NO_3)_2$), 600µl of potassium nitrate (KNO_3), 500µl of Fe-HEBD and 950mg of MES were added which act as a buffered medium. Magnet was added to the flask to mix the prepared solution in a magnetic stirrer. The pH of the solution was determined by the pH meter. The pH of the Hoagland medium is 5.7 and it was adjusted by adding 0.1N potassium hydroxide (KOH). The pH of the buffer was maintained and the solutions were transferred to two reagent bottles. Each of the bottles carries 500ml of liquid volume. Add 5g of type-a agar media to both bottles for solidification. 3ml of 30mM sodium chloride (NaCl) were added for salt treatment. Then, the bottles were kept for an autoclave.

1.3. Sowing the seeds on medium.

After completing the autoclave process, the medium was poured into square Petri plates in a laminar airflow chamber. The seeds were placed on a medium. Each plate was separated into two halves to place two different genotypes. Toothpicks were used to sow the seeds on the medium. The toothpicks were also autoclaved and not opened outside the chamber. The plates were sealed by tape after sowing. Then the plates are moved to the cold storage room and stored for 48 hours. After two days of storage in a cold room. The plants are moved to the growth culture room and allowed to stand on a rack. 14 days were the time period settled for the plant growth.

1.4. Image Analysis for the Root length (in cm).

The software program named Fiji win64 were used to generate scientific image analysis for 7-day and 14-day period intervals. The images were stacked, and the root lengths were calculated in pixels. Then, it was converted into root length in cm.

1.5. Harvesting the samples from the medium.

After 14 days of growth period, the shoot and root of Arabidopsis were harvested. The seals were removed using a blade. With the help of a small scissor the shoot and root samples were separated. They were transferred into Eppendorf tubes by using forceps. The tubes were quickly transferred into a liquid nitrogen vessel. Then the tubes with shoot and root samples were stored in a freezing refrigerator at -80 ℃.

1.6. Extraction of RNA.

The RNA isolation was performed by (Logemann et al., 1987). Liquid nitrogen was used to crush seedlings that were frozen. 400µl of Z6 buffer, 8M guanidinium-HCL, and 20 mM EDTA pH 7.0 were used to homogenize the samples. After, 400µl of phenol was added. For phase separation, samples were vortexed and centrifuged for ten minutes at 20,000 g. The aqueous phase was transferred to a separate tube, and 0.05 volumes of 1 N acetic acid and 0.7 volumes of 96% ethanol were added. The RNA precipitated at -20℃.

After centrifugation (20000g, 10 minutes, 4 degrees Celsius), the pellet was cleaned with 70% ethanol and 200µl of sodium acetate (pH 5.2). After drying, the RNA was dissolved in 30µl of ultrapure water and stored at -80 ℃ until use. Then the synthesized RNA concentrations were estimated by Nanodrop Spectrophotometers.

1.7. RevertAid first strand cDNA synthesis.

The stored RNA samples were thawed, and mixed. The components in the kit were centrifuged. The two Eppendorf tubes are labelled as sodium treatment and control. The specific gene named *Col.02x* was selected based on the RNA concentrations. A total volume of 20µl was prepared for cDNA synthesis. 9.9µl of (*Col.02x* –Na) and 11µl of (*Col.02x* –control) was taken as a template strand. To this, add 1µl of oligo (dT) primer in both the tubes and 1.01µl of nuclease-free water in the Na treatment tube alone and it makes 12µl of total volume in both the tubes. In both tubes, add 4µl of 5x Reaction Buffer, 1µl of RNase inhibitor, 2µl of 10mM dNTP mix and 1µl of RevertAid M - MUL – VRT (200U/ µl). Thus, the total volume is 20µl.

1.8. PCR.

Thermo Scientific™ RevertAid™ MyTaq cDNA Synthesis Kit also has a procedure for PCR amplification. 1µl of template strand were taken in two PCR tubes. 1µl of the known sample were taken as a positive control and 1µl of nuclease-free water were taken as a negative control. Master Mix has been prepared for five reactions. For the master mix, 25µl of 5x MyTaq Buffer, 2.5µl of MyTaq pol, 2.5µl of Forward primer and 2.5µl of Reverse primer were added and 87.5µl of nuclease-free water was added. Oligonucleotide primers are used for PCR reactions. The PCR reactions were performed for 25 thermocycles. The programs were set up as a first step for initial denaturation at 95℃ for one minute. The next step is as follows, Denaturation at 95℃ for 15 seconds, Annealing at 60℃ for 15 seconds and Extension at 72℃ for 20 seconds. After the process is completed, it can be stored at -20℃ for gel electrophoresis.

1.9. Agarose gel Electrophoresis.

1% of Agarose gel was prepared. 0.7g of Agar was taken in a beaker which was dissolved in 70ml of 0.5x TBE buffer. Then, it was heated in a microwave oven. 5µl of ethidium bromide were added and mixed well. Then, the gel was transferred to tray. It was allowed to withstand incubation for half an hour for solidification. After the time interval, the comb was removed. 5µl of the maker and 10µl of samples (templates-Na, con and positive & negative control) were added. The cathode and electrode were connected and set at 100v, 500mA for 50 minutes and allowed it to run (Lee et al., 2012).

1.10. Data analysis.

RSTUDIO (Desktop Version: 2024.04.2+764) were used for statistical analysis and package ggplot2 (Version 3.5.1) were used to generate graphs. "ThaleMine" version 5.1.0-20230710 was used for gene annotation. The gene Annotation can be performed by using ThaleMine. The PANTHER analyses tool was used for GO enrichment analysis where, gene ID has entered and the required organisms were selected. For an example, Arabidopsis thaliana and command the analysis need to be performed in statistical overrepresentation test. In the statistical overrepresentation test, GO biological process complete, GO cellular component complete and GO molecular function complete analysis were performed by selecting FISCHER test performance in settings and false discovery rate correction were observed. Bioinformatics and Evolutionary Genomics, was a web tool which were used to calculate the comparison of different groups and plot it as a Venn diagram. Input the genotypes and represent the name and select to plot a Venn diagram. The graphical output is in SVG and PNG format.

(https://bioinformatics.psb.ugent.be/webtools/Venn)

2. Results

A. RNA sequence analysis

The RNAseq data were obtained for Differential Expression Genes (DEG's) which are already calculated by utilizing the limma R package, comparing different conditions across various genotypes and treatments, such as low, medium, and high nitrate soils. These multiple contrasts aimed to assess the expression changes between specific conditions. Their statistical values are in log2 fold change (log2FC) of gene/transcript between the contrast groups. Our goal was to assess the DEG's data and to find the respective genes which are responding to a WGD event and how lack of K transporters affects and what soil treatment has been followed. From the list of DEG's dataset of shoot sample, the genes were sorted based on the contrast between diploid and neo-tetraploid mainly AKT1 and HAK5 as they are K+ transporters. The venn diagrams were generated by using an online tool. As the gene list entered in Input data and highlighted with title as missing *akt1* or *hak5*. Then, it will output image as a file which is shown in Figure1 and Figure2.

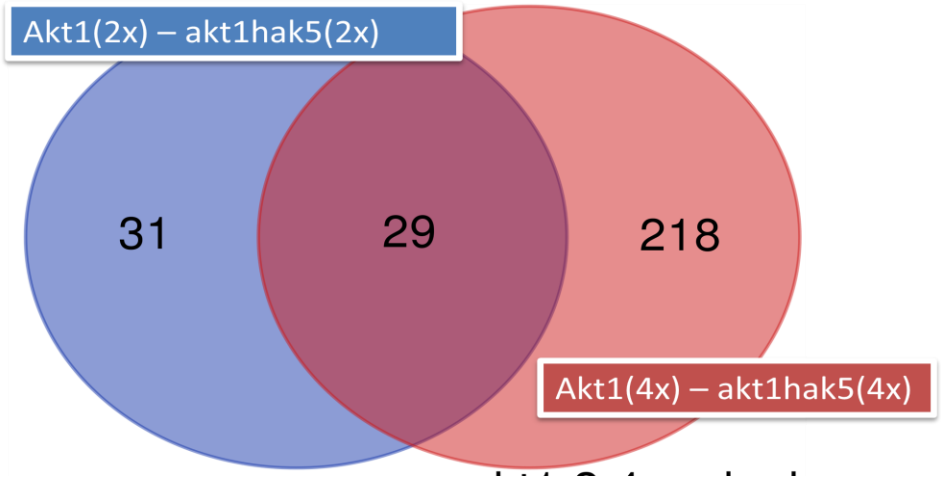

Figure1; *akt1* background which missing *hak5* gene, Venn diagram which was created by https://bioinformatics.psb.ugent.be/webtools/Venn website tools. The blue colour indicates contrast between diploid akt1 and akt1hak5. The pink colour indicates the contrast between tetraploid akt1 and akt1hak5.

From the venn data information **(Figure1)** it was observed that 29 genes share their common similarities between diploid vs tetraploid *akt1* genes and *akt1hak5* double genes. There are 31 different genes in *akt1* and double diploid and 218 different genes in tetraploid of *akt1* and double genes. The genes sharing common similarities were annotated by using ThaleMine and it was tabulated in Supplemental data **Table1.**

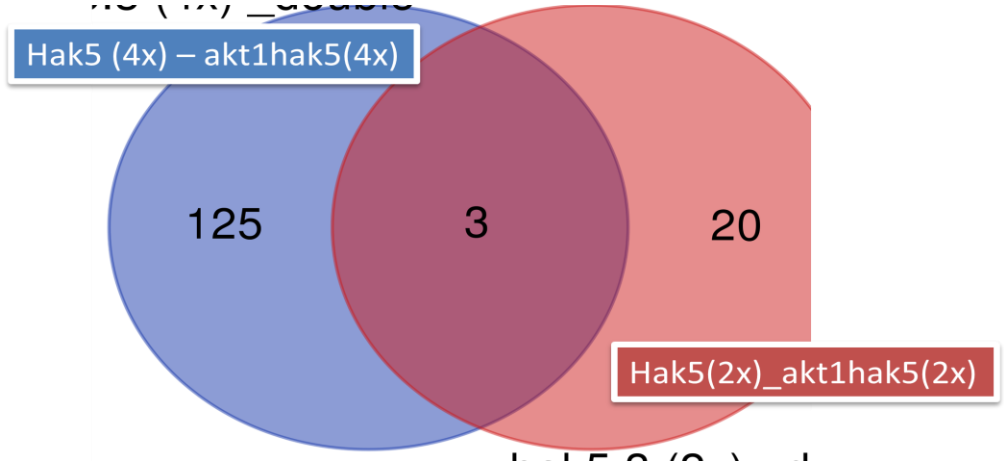

Figure2; *hak5* background which missing *akt1* gene. The blue colour indicates contrast between tetraploid *hak5* and *akt1hak5*. The pink colour indicates the contrast between diploid *hak5* and *akt1hak5*. From the venn data information (**Figure2**) it was observed that 3 genes share their common similarities between diploid vs neo-tetraploid *hak5* genes and *akt1hak5* double genes in the absence of *akt1* background. There are 20 different genes in *hak5* and double diploid and 125 different genes in tetraploid of *hak5* and double genes. The genes sharing common similarities were annotated by using ThaleMine and it was tabulated in Supplemental data **Table2.**

B. Gene Ontology Enrichment Analysis

In differential gene expression study, it's challenging to make sense of the biological implications of this gene list. Gene Ontology (GO) enrichment analysis helps by categorizing these *akt1* background which missing *hak5* genes and *hak5*

background which missing *akt1* genes into known biological processes, molecular functions, and cellular components, making it easier to interpret the data in a biological context. PANTHER is used for GO enrichment analysis.

In the *akt1* background which missing *hak5* genes list has no statistically significant biological and molecular data. The cellular components were tabulated in supplemental data **Table3.** In the cellular complete of GO enrichment analysis of *akt1* background which missing *hak5* genes list shows "nucleus" as a significantly enriched cellular component, it suggests that a large number of the differentially expressed genes (DEGs) in our study are associated with or localized to the nucleus of the cell. There are no significant results for *hak5* background which missing *akt1* genes list.

C. Gene Expression Analysis

The specific gene is selected out from the DEG's by plotting bar graph in Rstudio/ggplpot2. The gene has been chosen based on their log2FoldChange values and statistical p value. The gene observation noted for AT4G16860 in FPKM (fragments per kilobase of transcript per million mapped reads) which is shown in **Figure3**. The average gene expression (GE) value was high in *hak5* genotypes which are tetraploid in nature and it was low soil treatment. Followed by *hak5* mutant, the second highest GE FPKM value was observed in akt1hak5 diploid genotype. The mutant high soil treated tetraploid *hak5* tends to have a lower gene expression. The gene annotation has been taken from ThaleMine.

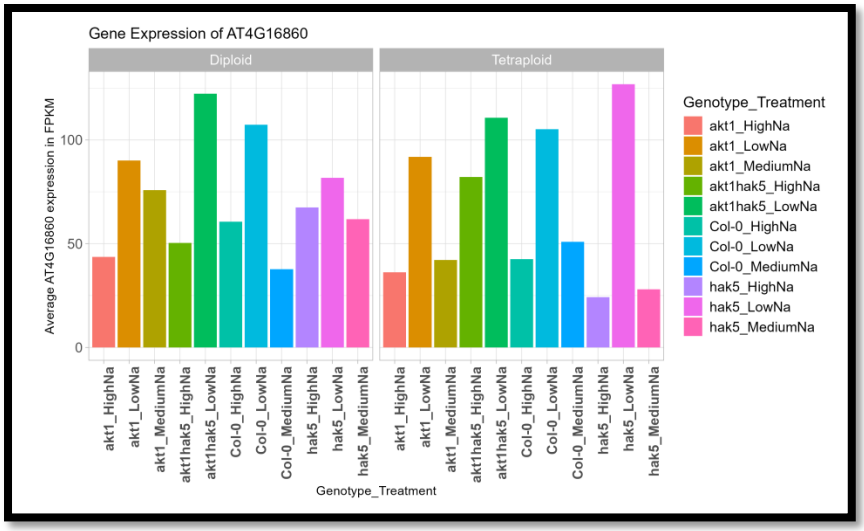

Figure3; Bar graph showing the gene expression for AT4G16860 for different genotypes (*Col-0* 2x, *Col-0* 4x, *akt1-2* 2x, *akt1-2* 4x, *hak5-3* 2x, *hak5-3* 4x, *akt1-2/hak5-3* 2x and *akt1-2/hak5-3* 4x) which undergoes three different soil treatment as low, medium and high. For the statistical analysis, Tukey's HSD (Honestly Significant Difference) post-hoc test, which were used after an ANOVA to determine which specific groups' means (compared with each other) are different which are tabulated in supplemental data **Table4.**

Tukey's HSD (Honestly Significant Difference) post-hoc test reveals most of the adjusted p-values are above 0.05, indicating that there are no statistically significant differences between the pairs of genotypes + treatment levels for the gene expression data as analyzed. However, the comparisons like *akt1hak5*_LowNa - *akt1*_HighNa (p adj = 0.0586912) and *hak5*_MediumNa - *akt1hak5*_LowNa (p adj = 0.0842490) are close to the significance threshold of 0.05, suggesting that there may be some trends worth further investigation. Supplemental data **Table4** also shows the comparison between tetraploid to diploid. The p-value is 0.7023, which is much higher than the typical alpha level of 0.05. This indicates that there is no statistically significant difference in AT4G16860 expression between the diploid and tetraploid plants. Based on the Tukey HSD test results, there is no significant difference in the expression of the "AT4G16860" gene between diploid and tetraploid genotypes. The difference

observed is likely due to random variation rather than a true effect of ploidy on gene expression.

The gene is recognition of peronospora parasitica 4 (RPP4) is coordinately regulated by transcriptional activation and RNA silencing (Huang et al., 2010). Moreover, RPP4 controls how plants react to temperature for both growth and survival (Balint-Kurti, 2019). For instance, mutants with active RPP4 genes may display symptoms associated with cell death, such as alterations in the morphology of chloroplasts and the build-up of ROS (Foyer, 2018).

D. Plant growth for gene expression analysis

Plant biology and the impact of genetic or environmental variables on gene expression can be better understood by examining gene expression in plants grown on plates in a controlled, effective, and scalable manner. Therefore, the plants were grown in a Hoagland medium. Their root length were calculated in cm and plotted as a graph by using Rstudio/ggplot2.

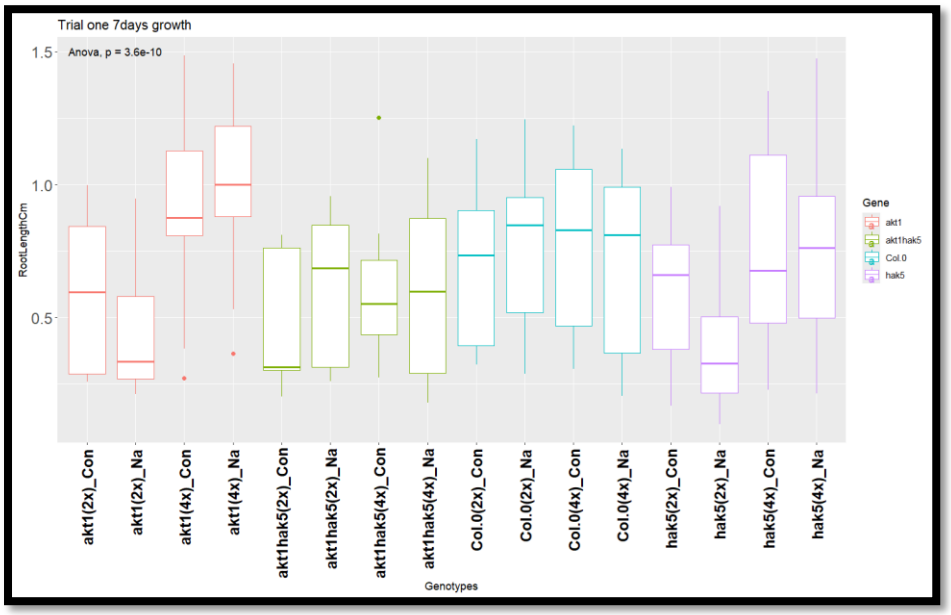

Figure4; Box plot showing Trial one 7days growth of the root length in cm for control and salt treated (*Col-0* 2x, *Col-0* 4x, *akt1-2* 2x, *akt1-2* 4x, *hak5-3* 2x, *hak5-3* 4x, *akt1-2/hak5-3* 2x and *akt1-2/hak5-3* 4x) genes. For the statistical analysis, Tukey's HSD (Honestly Significant Difference) post-hoc test, which were used

after an ANOVA to determine which specific groups' means (compared with each other) are different which are tabulated in supplemental data **Table5.**

From the observed **Figure4** shows Tukey's HSD (Honestly Significant Difference) post-hoc test adjusted p-values is 3.6e-10 which are extremely low, means that the differences in root length among the groups (defined by Genotype_Treatment and Gene) are statistically significant. In other words, there is a very strong likelihood that the observed differences are not due to random chance. Table5 in supplemental data shows the most significant results are related to comparisons involving *akt1*(4x)_Na and *hak5*(2x)_Na, which show a clear difference in means compared to other groups. Nevertheless, comparisons involving *hak5*(4x)_Na and *akt1*(4x)_Con also show significant differences.

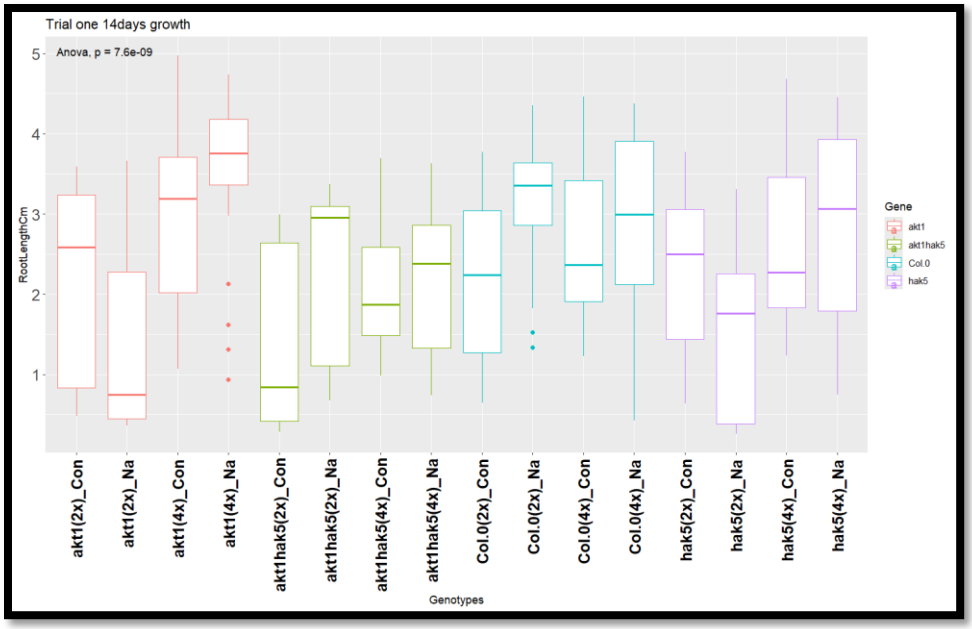

Figure5; Box plot showing Trial one 14days growth of the root length in cm for control and salt treated (*Col-0* 2x, *Col-0* 4x, *akt1-2* 2x, *akt1-2* 4x, *hak5-3* 2x, *hak5-3* 4x, *akt1-2/hak5-3* 2x and *akt1-2/hak5-3* 4x) genes. For the statistical analysis, Tukey's HSD (Honestly Significant Difference) post-hoc test, which were used after an ANOVA to determine the specific groups' means (compared with each other) are different which are tabulated in supplemental data **Table6.**

The ANOVA test yielded a p-value of 7.6e-09, indicating a very significant statistical difference between the genotype + treatment groups was shown in

Figure5. On the other hand, p- value of trial two 7days growth is < 2e-16 indicating significant statistical difference which was shown in **Figure6.**

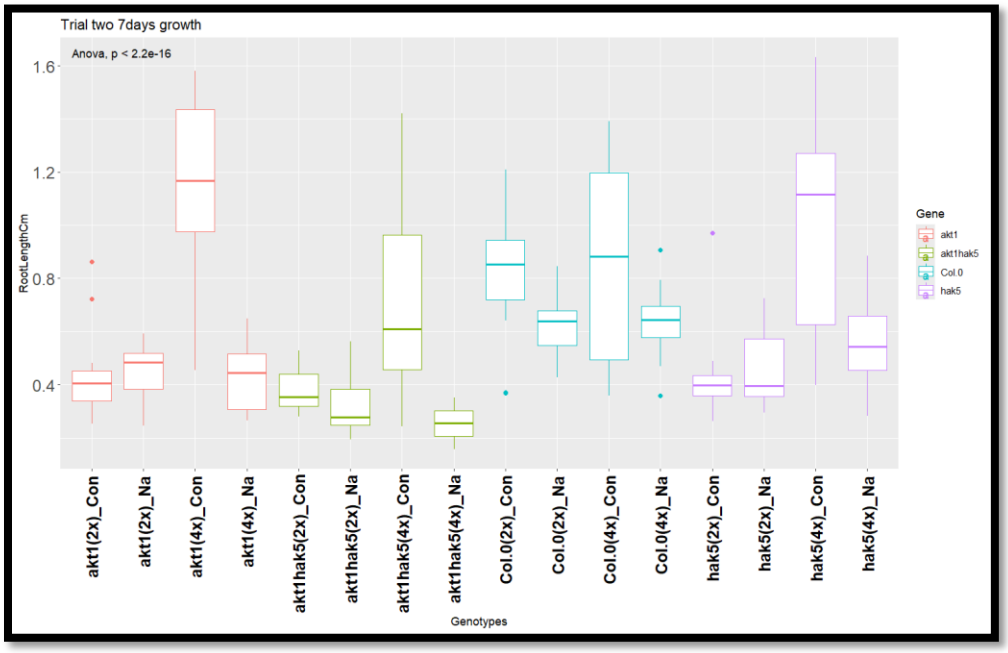

Figure6; Box plot showing Trial two 7days growth of the root length in cm for control and salt treated (*Col-0* 2x, *Col-0* 4x, *akt1-2* 2x, *akt1-2* 4x, *hak5-3* 2x, *hak5-3* 4x, *akt1-2/hak5-3* 2x and *akt1-2/hak5-3* 4x) genes. For the statistical analysis, Tukey's HSD (Honestly Significant Difference) post-hoc test, which were used after an ANOVA to determine the specific groups' means (compared with each other) are different which are tabulated in supplemental data **Table7.**

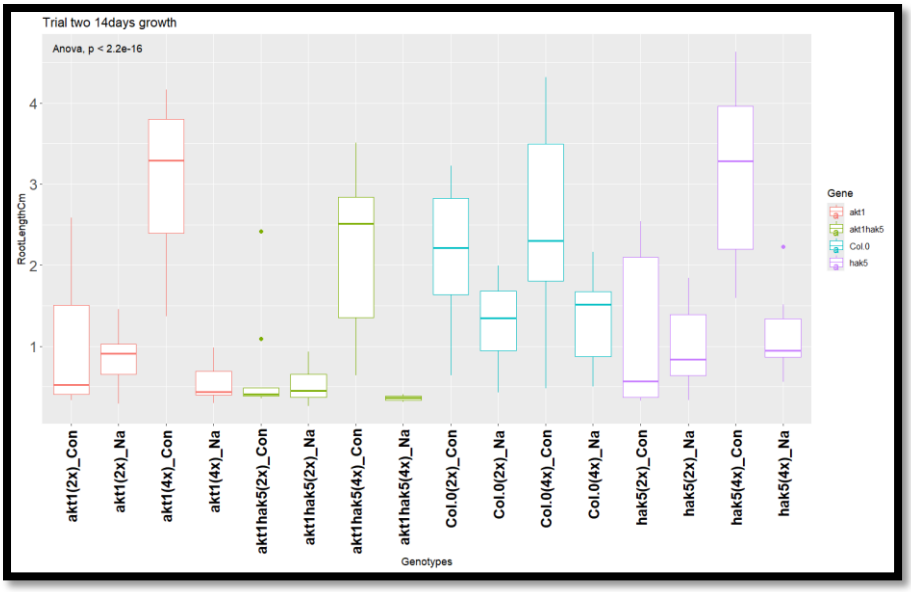

Figure7; Box plot showing Trial two 14days growth of the root length in cm for control and salt treated (*Col-0* 2x, *Col-0* 4x, *akt1-2* 2x, *akt1-2* 4x, *hak5-3* 2x, *hak5-3* 4x, *akt1-2/hak5-3* 2x and *akt1-2/hak5-3* 4x) genes. For the statistical analysis, Tukey's HSD (Honestly Significant Difference) post-hoc test, which were used after an ANOVA to determine the specific groups' means (compared with each other) are different which are tabulated in supplemental data **Table8.**

From the observed **Figure7** shows Tukey's HSD (Honestly Significant Difference) post-hoc test adjusted p-values is 2.2e-16 which are extremely low, means that the differences in root length among the groups are statistically significant..

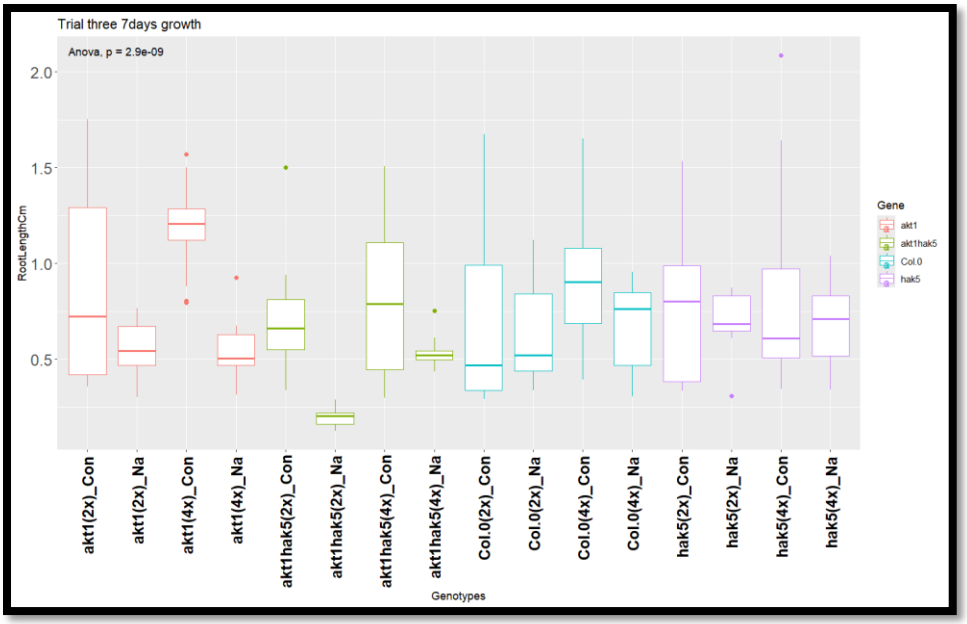

Figure8; Box plot showing Trial three 7days growth of the root length in cm for control and salt treated (*Col-0* 2x, *Col-0* 4x, *akt1-2* 2x, *akt1-2* 4x, *hak5-3* 2x, *hak5-3* 4x, *akt1-2/hak5-3* 2x and *akt1-2/hak5-3* 4x) genes. For the statistical analysis, Tukey's HSD (Honestly Significant Difference) post-hoc test, which were used after an ANOVA to determine the specific groups' means (compared with each other) are different which are tabulated in supplemental data **Table9.**

From the observed **Figure8** shows Tukey's HSD (Honestly Significant Difference) post-hoc test adjusted p-values is 2.9e-09 which are extremely low, means that the differences in root length among the groups are statistically significant.

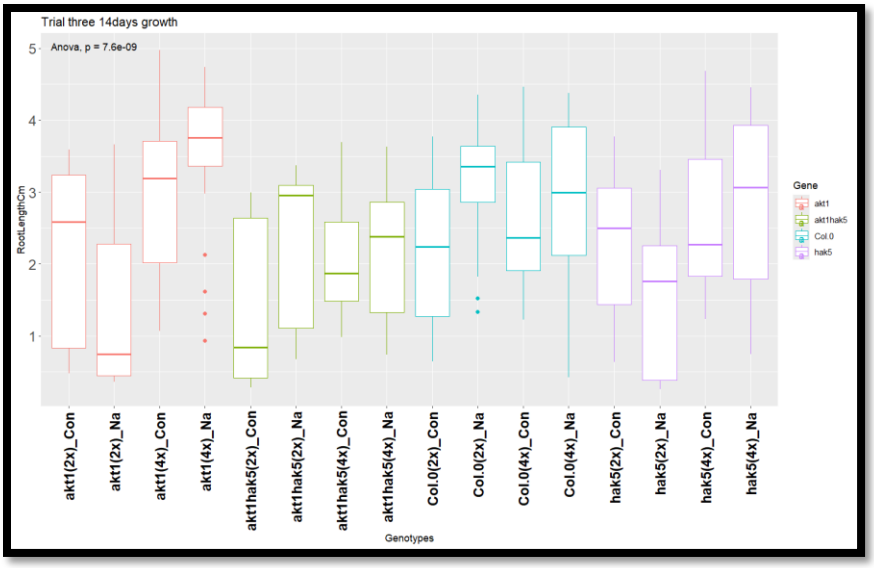

Figure9; Box plot showing Trial three 7days growth of the root length in cm for control and salt treated (*Col-0* 2x, *Col-0* 4x, *akt1-2* 2x, *akt1-2* 4x, *hak5-3* 2x, *hak5-3* 4x, *akt1-2/hak5-3* 2x and *akt1-2/hak5-3* 4x) genes. For the statistical analysis, Tukey's HSD (Honestly Significant Difference) post-hoc test, which were used after an ANOVA to determine the specific groups' means (compared with each other) are different which are tabulated in supplemental data **Table10.**

From the observed **Figure9** shows Tukey's HSD (Honestly Significant Difference) post-hoc test adjusted p-values is 7.6e-09 which are extremely low, means that the differences in root length among the groups are statistically significant.

The **table11** shows the concentrations of RNA for the shoot samples. The resultant values are not expected and they are very low. The scanty amount of nucleic acids is due to loss of RNA during extractions. Therefore, to select the control and sodium treatment genotypes, *Col.0*2x gene is chosen to observe gene expression. *Col.0*2x undergoes PCR reactions and gel electrophoresis.

Table11; The RNA Concentrations which was determined by Nanodrop spectrophotometer. These genotypes from the shoot samples are selected by random manner.

S. No	Genotypes	Nucleic Acid	Unit
1	Na – hak5(2x)	28.2	ng/µl
2	Na – Col.02x	27.1	ng/µl
3	Na – Col.04x	8.7	ng/µl
4	Na –akt1 (4x)	7.8	ng/µl
5	Na – akt1hak5 (2x)	27.8	ng/µl
6	Na – akt1hak5 (4x)	275.2	ng/µl
7	Na – hak5 (4x)	8.9	ng/µl
8	Con – akt1(4x)	53.6	ng/µl
9	Con – Col.02x	24.6	ng/µl
10	Con – akt1(2x)	48.5	ng/µl

E. Agarose gel electrophoresis

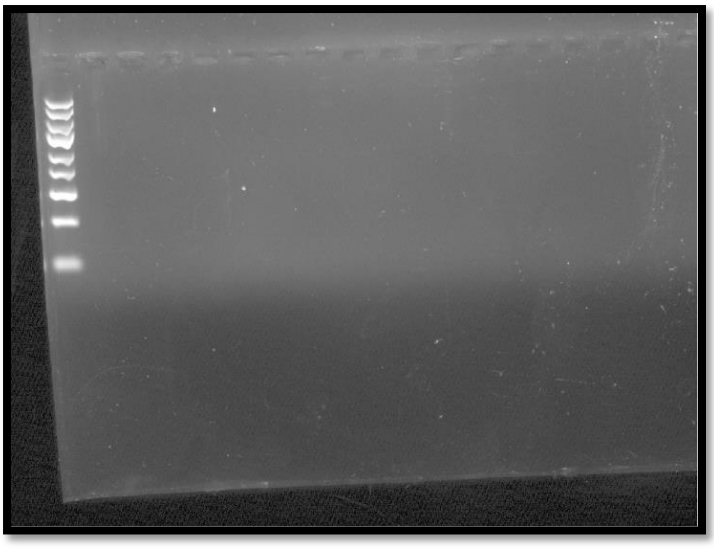

Figure10; Picture of Agarose gel electrophoresis for the *Col.0*2x – control and sodium treated shoot samples. This resultant band is from the marker. There is no band visualization from other samples.

The agarose gel electrophoresis is carried out for the genotype *Col.0*2x – control and sodium treated shoot samples. Furthermore, the positive control of previously generated cDNA and the negative control is nuclease – free water. However, there is no gene expression for all samples. The gel **Figure10** shows that the PCR reaction has failed or cDNA hasn't been synthesized successfully due to limited amount of RNA. Due to the time schedule, the procedure couldn't be repeated.

3. DISCUSSIONS

The molecular changes that occurs following whole-genome duplication (WGD) have not yet been thoroughly studied, leaving the phenotypic changes in neo-tetraploids largely unexplained. To address this, we investigated the gene expression in neo-tetraploids lacking key K+ transporters and plants were grown on sodium-rich and control environment. Our aim was to uncover the molecular mechanisms that enable neo-tetraploids to outperform diploids in saline environments (Chao et al., 2013). Our anticipation is that tetraploid plants might inhibit Na+ uptake or enhance their ability to store Na+ more effectively under salt stress conditions. Alternatively, they may increase their K+ uptake capacity to maintain ionic balance and mitigate the effects of salt stress. However, since no ionomic data has been collected, these hypotheses remain untested.

Our findings from the Venn diagram analysis showed the gene lists which sharing common similarities between diploid Vs tetraploid *akt1* genes Vs *akt1hak5* double genes & *hak5* Vs double genes. With these shared gene lists, we can perform further functional and pathway analysis to identify the molecular mechanisms that are consistently affected across these different genotypes. By examining the overlap, we can pinpoint genes and pathways that are likely involved in key processes such as K+ transport, ion homeostasis, and stress responses. This analysis could help us understand how whole-genome duplication (WGD) influences gene expression and how the lack of specific K+ transporters (AKT1 and HAK5) impacts these processes. The molecular mechanisms inferred from this analysis may include enhanced ion transport capacity, altered stress signaling pathways, and adaptive changes in gene regulation that allow tetraploids to better cope with saline environments compared

to diploids. This could suggest that tetraploid plants have evolved compensatory mechanisms to overcome the deficiencies in specific K+ transporters, contributing to their improved performance under stress conditions.

In the Gene Ontology (GO) enrichment analysis focused on the cellular component of the AKT1 background, where HAK5 is missing, the results showed a significant enrichment for the "nucleus" component. On the other hand, in the HAK5 background where AKT1 is missing, the GO enrichment analysis did not yield any significant cellular component results. This difference in enrichment patterns may point to distinct molecular mechanisms by which these two K+ transporters influence cellular processes. Specifically, AKT1 may play a more direct or influential role in processes that are regulated or coordinated within the nucleus when HAK5 is absent, highlighting the complexity of gene regulation and the functional interplay between these transporters.

Overall, the molecular mechanisms of *RPP4* involve a tightly regulated balance between activating defense responses and preventing excessive damage to the plant itself. The gene's regulation by transcriptional activation and RNA silencing, its role in temperature-dependent growth responses, and its involvement in controlling cell death and ROS accumulation all contribute to the plant's ability to effectively respond to environmental stresses and pathogenic attacks.

It was anticipated that root length would be reduced compared to the control, as Na+ typically inhibits the potassium uptake system in both wild-type and mutant plants. However, in our study, we observed that the growth of Arabidopsis roots and shoots under salt stress conditions was similar to that of the control. This outcome suggests that the salt concentration used may not have been sufficient to significantly impact plant growth, as expected.

The RNA sample was extracted but it is not due to expected values. This can happen due to various reasons overloading a column might jam it or hinder RNA from binding effectively (Vermeulen et al., 2011). If the extraction process has gone smoothly, we would have anticipated obtaining a high-quality RNA sample with sufficient yield for downstream applications. This would have allowed for accurate and reliable quantification of gene expression, facilitating the study of gene

regulation under various conditions. The high-quality RNA would have been instrumental in producing clear and interpretable data, essential for understanding the biological processes under investigation.

The selection of genes for the primer design is complicated due to the duplication of genes. It was sorted from the gene list that AT4G16860 – RPP4 is suitable for primer design. Leucine-rich repeat proteins with Toll/interleukin-1 receptor domains that bind nucleotides are encoded by the RPP5 multigene family, of which RPP4 is a member and so the nucleotide sequence between AT4G16950 (RPP5) and AT4G16860 (RPP4) are mostly similar (Van Der Biezen et al., 2002). Thus, it will not be suitable for primer design. Due to a shortage of schedule, primer designing can't be further carried out. Due to this sequence similarity, designing primers specific to AT4G16860 (RPP4) is problematic. The primers could potentially bind to AT4G16950 (RPP5) as well as AT4G16860, leading to non-specific amplification. As a result, the PCR reactions would produce mixed or ambiguous results, making it difficult to determine which gene is being amplified. This non-specific amplification would compromise the accuracy of gene expression studies, leading to unreliable data. Therefore, AT4G16860 (RPP4) is not suitable for primer design when such sequence similarities exist.

From the observed RNA reading, the *Col.0*2x gene has been selected based on its control and salt treatment values. However, the gene expression for this gene hasn't been done due to technical error. The PCR reactions were not successful due to improper handling. It may also cDNA synthesis was not successful (Lorenz, 2012). Therefore, agarose gel electrophoresis results only in the control marker bands. The PCR reactions intended to assess the gene expression of the *Col.0*2x gene were not successful due to technical errors during handling, which led to unsuccessful agarose gel electrophoresis results. Had the procedure been successful, we would have demonstrated that the plate system is well-suited to reveal differences in gene expression, comparable to those observed in a soil system. These differences in gene expression could have been attributed primarily to the sodium content in the soil. However, if sodium was not the primary factor, other soil parameters, such as clay content, ion concentrations, or pH levels, could also have contributed to the

observed variations in gene expression (Gupta et al., 2024). Due to the time limit the procedure hasn't repeated.

4. Conclusion

In our study, we observed no major difference in K+ uptake between diploids and neo-tetraploids. This comparison study is made randomly to know the difference between diploid and neotetraploid K+ uptake incase lack of transporters like AKT1 and HAK5 and observing tolerance to salt. Akt1 and Hak5 or their regulators are not suppressing the elevation of leaf K+ in neotetraploids caused by whole genome duplication (Fischer et al., 2022). Still, there is a knowledge gap in the molecular study. Furthermore, a detailed comparison of gene set data and repeating the PCR and gene expression procedure might be able to study a beneficial effect of whole–genome duplication without any drawbacks. Thus, it will provide a way to develop the crops more applicable to stress tolerance.

Supplemental data

- **Table1; ThaleMine Gene Annotation for *akt1* background which missing *hak5.*** Gene Annotation for akt1 background which missing hak5.xlsx
- **Table2; ThaleMine Gene Annotation for *hak5* background which missing *akt1*.** Gene Annotation for hak5 background which missing akt1.xlsx
- **Table3; PANTHER GO Enrichment Analysis** GO enrichment analuysis.xlsx
- **Table4; Tukey (HSD) multiple comparisons of means for Gene Expression of *RPP4.*** Tukey (HSD) multiple comparisons of means for Gene Expression of RPP4.xlsx
- **Table5; ANOVA Analysis for Trial one 7days growth** ANOVA Analysis for Trial One 7days growth.xlsx
- **Table6; ANOVA Analysis for Trial one 14days growth** ANOVA Analysis for Trial One 14days growth.xlsx
- **Table7; ANOVA Analysis for Trial two 7days growth** ANOVA Analysis for Trial two 7days growth.xlsx
- **Table8; ANOVA Analysis for Trial two 14days growth**

ANOVA Analysis for Trial two 14days growth.xlsx

- **Table9; ANOVA Analysis for Trial three 7days growth**

  ANOVA Analysis for Trial three 7days growth.xlsx

- **Table10; ANOVA Analysis for Trial three 14days growth**

  ANOVA Analysis for Trial three 14days growth.xlsx

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