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FUNCTIONAL CHARACTERIZATION OF POTATO ARP1 GENE ENCODING AUXIN-REPRESSED PROTEIN

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FUNCTIONAL CHARACTERIZATION OF POTATO ARP1 GENE ENCODING AUXIN-REPRESSED PROTEIN IN ARABIDOPSIS THALIANA UNDER SALANITY STRESS CONDIDTION

Sara Ali Abdulla Maleeh Alneyadi

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Cover: The effect of salinity stress conditions on transgenic *Arabidopsis* plants expressing *ARP1*. From left to right: Wildtype under salinity stress, *ARP1* and *ARP2* under salinity stress.

(Photo: By Sara Ali Abdulla Maleeh Alneyadi)

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Declaration of Original Work

I, Sara Ali Abdulla Maleeh Alneyadi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Functional Characterization of Potato Arp1 Gene Encoding Auxin-Repressed Protein in Arabidopsis Thaliana Under Salanity Stress Condidtion"*, hereby, solemnly declare that this is the original research work done by me under the supervision of Dr. Mayank Gururani, in the College of Science at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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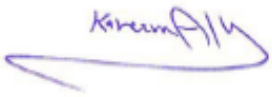
Signature  Date: 21/6/2024

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Abstract

Auxin-repressed proteins (ARPs) are conserved in higher plants and are involved in plant growth anddevelopment by controlling gene expression. ARP1 has been studied for its role in biotic stress response, but its role in abiotic stress response remains unclear. In this study, transgenic *Arabidopsis* plants expressing potato (*S. tuberosum)* ARP1 were evaluated for their salinity stress response. By gradually increasing NaCl concentrations from 0 to 200mM, plants were subjected to high salinity stress. As compared to wild-type (WT) control plants under stressed conditions, the ARP1 transgenic plants demonstrated improved height and root length and higher chlorophyll content. Under NaCl-induced salinity stress, the stomatal conductance of ARP1 plants was higher than that of WT plants. These measures indicate stress-related tissue damage and plant water status, respectively. As compared to WT plants, ARP1 plants accumulated more proline. Genes encoding antioxidant enzymes such as, Ascorbate peroxidase (APX), Superoxide dismutase (SOD) and Catalase (CAT) were expressed at higher levels in ARP1 plants, indicating better Reactive oxygen species (ROS) detoxification capacity. Chlorophyll-a fluorescence kinetics analyses showed that overexpression of the *S. tuberosum* ARP1 gene increased PI_{ABS} and PI_{total} indices as well as quantum yields and efficiency of photosystem II (PSII) as measured in eleven critical photosynthetic parameters, on salinity stressed ARP1 plants. Overall, this study has exhibited the positive role of potato auxin-repressed protein in alleviating salinity stress tolerance in higher plants.

Keywords: Applied mathematics, delay differential modelling, HIV, tumor-immune interaction, optimal control, bifurcation, chemo-immunotherapy.

Title and Abstract (in Arabic)

التوصيف الوظيفي لجين البطاطس *ARP1* الذي يشفر بروتين اكسن المكبوت في نباتات الاربدوبسيس تحت ظروف إجهاد الملوحة

الملخص

يتم حفظ بروتينات الأوكسين المكبوتة (ARPs) في النباتات العليا وتشارك في نمو النبات وتطوره من خلال التحكم في التعبير الجيني. تمت دراسة ARP1 لدوره في الاستجابة للإجهاد الأحيائي، لكن دوره في الاستجابة للإجهاد للأحيائي يظل غير واضحًا. في هذه الدراسة، تم تقييم نباتات الأرابيدوبسيس المعدلة وراثيا التي تعبر عن البطاطس ARP1 من حيث استجابتها لإجهاد الملوحة. من خلال زيادة تركيزات كلوريد الصوديوم تدريجيا من 0 إلى 200 ملم، تعرضت النباتات لإجهاد ملوحة عالية. بالمقارنة مع محطات التحكم من النوع البري (WT) في ظل الظروف المجهدة، أظهرت النباتات المعدلة وراثيا ARP1 تحسنًا في الارتفاع وطول الجذر ومحتوى أعلى من الكلوروفيل. تحت إجهاد الملوحة الناجم عن كلوريد الصوديوم، كانت التوصيلية الفمية لنباتات ARP1 أقل بكثير من تلك الموجودة في نباتات WT. تشير هذه التدابير إلى تلف الأنسجة المرتبطة بالإجهاد وحالة المياه النباتية، على التوالي. بالمقارنة مع WT، تراكمت ARP1 كمية أكبر من البرولين. تم التعبير عن بيروكسيداز الأسكوربات (APX) وديسموتاز الفائق أكسيد (SOD) والكاتلاز (CAT) بشكل متكرر أكثر في نباتات ARP1، مما يشير إلى قدرة أفضل على إزالة السموم من ROS. أظهرت تحليلات حركية الكلوروفيل-أ أن الإفراط في التعبير عن جين *S.tuberosum* ARP1 أدى إلى زيادة مؤشرات أداء $PIA_{PItotal}$ و PI_{ABS} وPItotal بالإضافة إلى إنتاجية الكم وكفاءة النظام الضوئي PSII كما تم قياسه في أحد عشر معلمة مهمة للتمثيل الضوئي، على نباتات ARP1 المجهدة بالملوحة.

مفاهيم البحث الرئيسية: اربدوبسيس، اوكسين، كلوروفيل أ، الهورمونات، البناء الضوئي، ملوحة، المعدلة وراثيا.

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Dedication

To my beloved parents and family

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List of Abbreviations

APX	Ascorbate Peroxidase
CAT	Catalase
MDA	Malondialdehyde
PSII	Photosystem II
qRT-PCR	Quantitative Real Time-PCR RBP
ROS	Reactive Oxygen Species

Chapter 1: Introduction

1.1 Overview

One of the primary abiotic stresses is rising soil salinity, which has a detrimental effect on plant development and growth. In 100 countries, there are currently approximately one billion hectares (ha) of land affected by soil salinity issues, and this amount is projected to increase by 0.3–1.5 million ha annually (Dhankher & Foyer, 2018). The worst hit are dry and semi-arid regions, such as those in the United Arab Emirates. Numerous causes contribute to the region's growing salinity problem, such as insufficient precipitation, high rates of evaporation, scarcity of water supplies, and poor management of irrigation. As a result, one of the biggest challenges facing modern agriculture is reducing salt stress in plants (Munns & Tester, 2008). It will take more advancements in crop agronomic traits to solve the salt issue. Osmotic stress results from soil salinity's first-phase reduction of soil water potential (Munns & Tester, 2008). According to a previous study Chakraborty et al. (2018), too much salt in the soil prevents plants from absorbing water and other nutrients that are soluble in it, like K^+ and Ca^{2+} ions, which are necessary for plant growth. Second, depending on the level of salt stress, the second phase will begin within a few days or weeks where Cl^- and Na^+ ions begin to build up (ion toxicity) in different plant tissues. Over-absorption of Na^+ and Cl^- ions causes nutritional imbalances, metabolic and physiological disorders, membrane disruption, and an increase in reactive oxygen species (ROS) production (Gill & Tuteja, 2010). These effects collectively impair essential plant cellular functions (Chakraborty et al., 2018). To adapt to salinity, plants have evolved several strategies, such as producing osmoprotectants as proline, which is used to preserve the homeostasis of ion, triggering both antioxidant enzymatic and non-enzymatic, and adjusting different signaling molecules, such as hormones (Gururani, et al., 2015). Furthermore, salinity stress exacerbates crucial physiological traits in plants, including stomatal conductance, photosynthesis, seed germination rate, chlorophyll content of leaf, and other growth-related attributes (Bistgani et al., 2019; Karumannil et al., 2023). Under salt stress, chloroplasts, the cell organelles responsible for photosynthesis, become highly susceptible to damage. The increased salt concentration disrupts membranes, blurs the distinction between grana and stroma lamellae, causes thylakoid disorganization, and may

even lead to chloroplast breakdown (Hameed et al., 2021). Furthermore, salt stress leads to sodium (Na^+) and chloride (Cl^-) ion accumulationwithin chloroplasts, resulting in reduced plant water potential, impaired photosynthesis, and disturbances in electron transport (Vickers et al., 2009). Like other abiotic stressors, salt-induced stress enhances the activity of the enzyme chlorophyllase and/or generates excessive ROS, destabilizing the pigment protein complex and reducing photosynthetic pigments (Hasanuzzaman et al., 2017). It has been established that salt stress affects the expression of many genes that are essential for the formation and upkeep of normal chloroplast structure, which is necessary for photosynthesis. This leads to aberrant chloroplast structure. Huang et al. (2019) found that the expression of *Ndhf* genes linked to the light response, *Rbcl* genes linked to the dark response, and *Matk* genes connected to chloroplastic intron splicing was downregulated in *Eucalyptus robusta* chloroplasts during 150 mM NaCl stress (Huang et al., 2019). Chloroplasts were deformed and photosynthesis was decreased because of this downregulation. Because they regulate gene expression, auxin-repressed proteins (ARPs), which are preserved in higher plants, that promote growth and development. Several plant species express ARP1 both in dormant buds and non-growing tissues (Souza et al., 2019). The *ARP* gene family includes glycine-rich proteins and proteins linked to dormancy. According to Lee et al. (2013), the growth-stimulating phytohormone auxin controls several processes related to plant growth and development, including as vascular differentiation, apical dominance, lateral root formation, shoot elongation, and embryo patterning.

1.2 Statement of the Problem

According to a previous study, silencing *GERI* (Growth enhancement and resistance impairment) /*ARP1* increased the vulnerability of plants to infection by the tobacco mosaic virus, *Pectobacterium carotovorum* subsp. carotovora, and *Phytophthora parasitica* var. Nicotianae (Souza et al., 2019). While the functions of this gene in plant growth, development, and biotic stress resistance have been studied, it's unclear how it contributes to abiotic stress tolerance. This study aimed to functionally characterize ARP transgenic Arabidopsis lines under salinity stress conditions, employing detailed physiological, molecular, and biochemical approaches.

1.3 Relevant Literature

1.3.1 Abiotic Stresses in Plants

Abiotic stresses are important limiting variables that have an impact on agricultural productivity in terms of both quality and quantity (Gill & Tuteja, 2010). The demands of an ever-increasing human population combined with climate change are likely to make these risks much more serious. Drought, high salinity, extreme temperatures, inadequate or excessive water, ultraviolet radiation (UV) and heavy metals are some of the non-living factors that negatively impact living organisms in a specific environment. Globally, the combined effect of these stresses results in a significant decline in agricultural productivity. Plants must develop different strategies for stress-tolerance in order to avoid adverse effects of stress. Some of these strategies include osmoregulation, morphological adaptations, and enriched antioxidant activity. Plants use these strategies in order to cope with unfavorable environmental conditions (Gururani, et al., 2015).

1.3.1.1 Global Scenario of Salt Stress

Salt stress is one of the most challenging environmental factors that limits productivity of crops. Plants are sensitive to salinity, and when NaCl levels exceed 200 mM (El Sabagh et al., 2019), most cannot survive. It is estimated that over a billion hectares of land are impacted in more than 100 countries around the world, (Atta et al., 2023). According to the FAO, 73% of the land studied so far has been classified as salt-affected soil, which includes the topsoil (0-30 cm) accounts for 424 million hectares, whereas subsoil (30-100 cm) accounts for 833 million hectares (FAO, 2021). Soil salinity is substantially impacting crop productivity, especially in arid and semiarid regions (S. Hussain et al., 2019). In these regions, climate often serves as the primary catalyst for salinization, as evaporation leads to a gradual accumulation of salts. Inadequate irrigation methods and ongoing climate variability have resulted in heightened salt levels, known as salinity stress (S. Hussain et al., 2019; Negacz et al., 2022). Furthermore, the agricultural sector projected an annual loss of 27.3 billion US dollars due to agricultural damage caused by saline soils (Kumar & Sharma, 2020). Consequently, in addition to the outcomes of global warming and other environmental factors, the existence of excessive salts in soil poses a significant obstacle to global food security amidst the burgeoning world population.

1.3.1.2 The Effects of Salinity and Ions on Plants

In most plant stages, including seed germination, vegetative growth, and reproduction, salinity has an impact. Moreover, salinity stress intensifies critical physiological traits in plants, such as stomatal conductance, leaf chlorophyll content, photosynthesis, and other growth-related attributes (Bistgani et al., 2019). Salinity affects plant growth mechanisms in two ways: through water relations as well as through ionic relations (Brini et al., 2007). Salinity stress and consequent damage to plants can result from the excessive buildup of soluble ions (such as Na^+, Ca^{2+}, K^+, Mg^{2+}) in the root zone (Atta et al., 2023). The plant cell requires a specific concentration of ions like Na^+, K^+, and $Ca^{2+}$and any changes or imbalances in theses can result on destabilization of metabolic processes. Increased Na^+ accumulation inhibits nitrate reductase activity, which leads to photosystem II and thus chlorophyll breakdown (Gill & Tuteja, 2010). Moreover, if Na^+ is replaced with Ca^{2+}, the membrane function is negatively affected, leading to a higher level of leakiness.

High chloride accumulation has been extensively studied for its negative effects on plant cells, but it's still unclear how chloride toxicity occurs (Suzuki et al., 2012). The accessible reports suggest that extreme levels of Cl^- within the leaf tissues might inhibit nitrate reductase activity, which in turn effects photosynthetic function. In addition, dehydration and cell death are also caused by increasing salt accumulation in the intercellular space. Further improvements to crop cultivars can also be achieved by understanding how salinity impacts metabolic processes, particularly photosynthesis (Akilan et al., 2019). Overall, the adverse effects of salinity on plant growth include soil compactness and hardness, which make it difficult for plants to establish effective root systems. There are three types of stress caused by less water availability: osmosis stress, nutritional deficiencies because of decreased uptake of nutrients such as nitrogen, phosphorus, potassium, and calcium due to salinity; and ion toxicity (Figure 1).

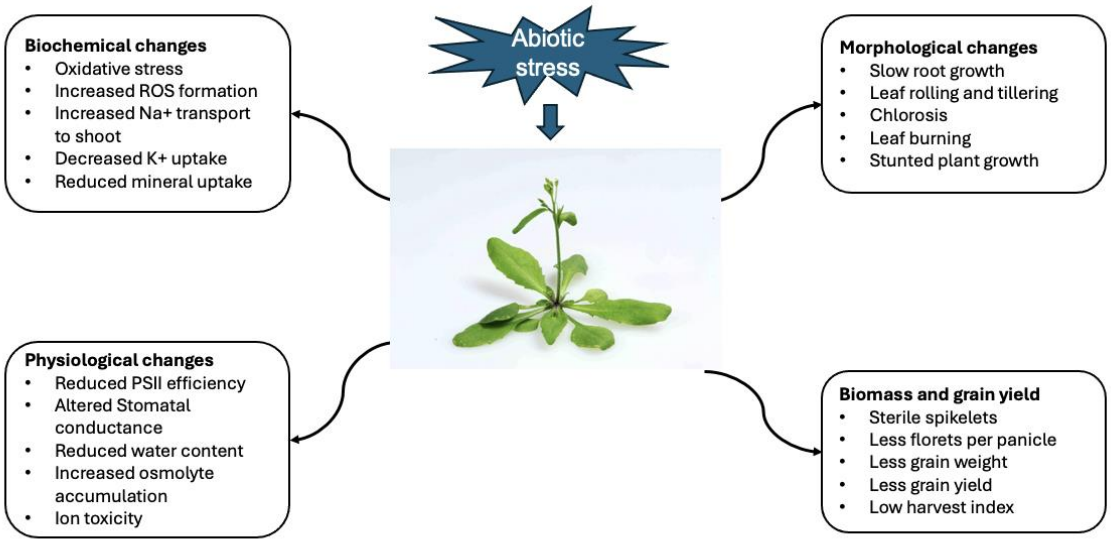

Figure 1: Effect of salinity stress to plant. Adapted from (Razzaq et al., 2019).

1.3.1.3 Biological Effects of Salinity on Water Intake, Osmotic Stress, and Nutrient Deficiencies

Salinity exerts biological effects by altering water intake mechanisms, inducing osmotic stress due to the imbalance in salt concentrations, and exacerbating nutrient deficiencies through impaired uptake and utilization. Soil salinity is commonly assessed based on Na^+ and Cl^- concentrations (Savvides et al., 2016). Plants are impacted by salinity in three primary ways. Firstly, the low osmotic potential of salt hampers water extraction from the soil, exposing plants to osmotic pressure, thereby limiting development and reducing yields. Secondly, excessive absorption and buildup of Na^+ and Cl^- ions in plant tissues from the soil lead to cytotoxicity, rising symptoms like leaf firing, diminished growth, and ultimately plant death. Additionally, elevated Na^+ levels reduce the accessibility of other ions for example Mg^{2+}, K^+ and Ca^{2+} due to cation competition, potentially leading to nutrient insufficiencies (Atta et al., 2023; Munns & Tester, 2008).

Excessive ions in soil water reduce the solute potential (ψs) and consequently the total water potential (ψw) of the soil. To maintain water uptake and turgor under such circumstances, plants must maintain their internal water potential (ψw) lower than that of the soil (Atta et al., 2023). Osmotic adjustment, facilitated by the accumulation of osmolytes such as organic acids, sugars, and amino acids in plant cells under salt stress,

helps plants reduce their water potential to enhance water uptake from saline soils (Hussain et al., 2022). In order to achieve this, plants either absorb salts and compartmentalize them inside their tissues or synthesize organic solutes. Salt-absorbing plants are known as halophytes. They are able to store salt concentrations in their tissues without disrupting their function. Alternatively, glycophytes plants do not absorb excess salt because they tolerate less salt concentrations in their tissues before they affect cell processes (Mutava et al., 2015). The majority of plants combine these strategies, with some diversities using different strategies.

An imbalance of nutrients between soil and plants is directly influenced by salinity. Sodium and chloride ion accumulation negatively impacts plant tissues and soil, affecting nutrient uptake. High Na concentrations antagonize potassium, and nitrogen absorption decreases under high salinities (Hussain et al., 2018). Saline environments are deficient in essential nutrients such as calcium, potassium, phosphorus, iron, and zinc. Under saline conditions, calcium and phosphate ions precipitate, reducing phosphorus uptake (Gill & Tuteja, 2010). Certain ion, including Na^+, B^-, and Cl^-, become toxic to plants. Rapid osmotic stress ensues when Na^+ accumulates in cell walls. Even at lower salinity levels, plants sensitive to particular toxic ion can suffer from sufficient quantities in the soil (Shrivastava & Kumar, 2015).

1.3.1.4 Effect of Salinity on Photosynthesis

Photosynthesis stands as the fundamental and vital physiological process driving plant growth and development, serving as the primary energy source for green plants. Salt stress significantly disrupts photosynthesis in plants (Gururani, 2022; Kappachery et al., 2024; Karumannil et al., 2023). The electron transport chain (ETC) is over-reduced in response to abiotic stress, which leads to photooxidation. Additionally, it causes damage to plant photosynthetic machinery (Zhao et al., 2021). The photosynthetic machinery depends heavily on Photosystem II (PSII) to sustain the effect of abiotic stress. Moreover, abiotic stress can damage the photosynthetic apparatus in addition to producing ROS as result of the imbalanced that happened in the redox signaling pathway. This inhibits PSII repair, which results in photoinhibition. As a way to overcome this problem, the extremely absorbed light is converted into thermal energy by plant, thus reducing the rate of electron

transport (Sasi et al., 2018; Zhao et al., 2021). According to an earlier study that examined how salinity affected the water potential in plant and photosynthesis in species called Kansas salt marsh, photosynthesis decreased in all species when the salinity was high, and stomata were specifically susceptible to an increase in the salinity in *T.ramosissima* (Betzen et al., 2019).

Under salt stress, chloroplasts, the cell organelles responsible for photosynthesis, are highly vulnerable to damage. The escalated salt concentration disrupts membranes, blurs the distinction between grana and stroma lamellae, causes thylakoid disorganization, and may even lead to chloroplast breakdown (Hameed et al., 2021). Accumulation of abundant Na^+ and Cl^- within leaves triggers water loss from guard cells resulting in reduced plant water potential, consequently altering the morphology, activity, and/or density of guard cells and stomata. These alterations can impede or even obstruct CO_2 entry, thereby impairing photosynthesis, and disturbances in electron transport from PS II to PS I (Mirfattahi & Eshghi, 2023; Vickers et al., 2009). Under salinity, plant cells that are experiencing osmotic stress absorb inorganic ions from the surrounding environment in addition to producing organic osmolytes like sorbitol, mannitol, proline, glycine, betain, polyamines, etc., the majority of which are found in the chloroplast (Betzen et al., 2019; Hameed et al., 2021). Similar to other abiotic stressors, salt-induced stress increases the activity of the enzyme chlorophyllase and/or produces too many ROS, which destabilises the pigment protein complex and reduces photosynthetic pigments (Hossain et al., 2019).

Some genes crucial for the development and maintenance of normal chloroplast structure, vital for photosynthesis, have been identified, and salt stress has been demonstrated to influence the expression of these genes, resulting in abnormal chloroplast structure. Huang et al. (2019) observed that under 150 mM NaCl stress in *Eucalyptus robusta* chloroplasts, the dark response was associated with *Rbcl,* the light response was associated with *Ndhf,* and the cholorplastic intron splicing that is related with *Matk* gene was downregulated. This downregulation led to reduced photosynthesis and distorted chloroplasts.

1.3.1.5 Increased ROS Production Under Salt Stress

Apart from affecting plants directly, salinity often leads to an extreme accumulation of ROS. The endoplasmic reticulum, mitochondria, cytosol, peroxisome, and chloroplasts

are the main organelles that generate ROS (Choudhury et al., 2017). As a result of salt-induced stomatal closure, leaves become less capable of storing carbon dioxide, which inhibits photosynthetic processes. A decline in photosynthesis rate leads to increased production of ROS and triggers enzymatic antioxidant responses, including superoxide dismutase (SOD), catalase (CAT), and various peroxidases (Jaspers & Kangasjärvi, 2010). These ROS can then interact with other vital plant cell components to cause oxidative damage in plants, including lipid peroxidation, DNA damage, protein oxidation, enzyme inactivation, hormone imbalances, and nutritional imbalances (Atta et al., 2023; Gururani, et al., 2015).

Aside from negatively affecting plant metabolism,, the complex nature of salt stress and water deficiency also produce ROS that have an adverse effect on plant systems. Studies on Chinese bayberry trees revealed significant increases in SOD, APX, and CAT activities under oxidative stress (Wu et al., 2012). Rice seedlings under salt stress were similarly shown to have greater H_2O_2 levels, higher MDA, and overproduced MG (methylglyoxal) Additionally, there was a rise in SOD and Lipoxygenase (LOX) activity and a reduction in CAT activity (Rahman et al., 2021). A study published in 2018 found significant increases in APX and CAT activities in diluted seawater treated sapodilla rootstock (Mohammadi et al., 2018). Another findings suggest that antioxidant agents can play a pivotal role in salinity tolerance mechanisms in date palm cultivars, with salinity-tolerant varieties exhibiting increased accumulation of both enzymatic and non-enzymatic antioxidants in leaf and root tissues in response to salinity stress (Al Kharusi et al., 2019). Salt stress induces ionic, osmotic and oxidative stress on chloroplasts, negatively impacting their function by reducing carbon assimilation and photosynthetic electron transport, consequently increasing ROS production (Wang et al., 2024).

1.3.1.6 Assessing the Photosynthesis Effects of Stress Using Chlorophyll-a Fluorescence

Photosynthetic specimens are subjected to chlorophyll a fluorescence induction when they are moved from darkness to light. Two phases exist in fluorescence induction: (i) the fast induction phase, which is also known as the OJIP, where O stand for origin, P stand for peak, and J=intermediate and I stand for inflection point, PSMT, where P means peak, S

means steady, M means maximum, and T means terminal (Figure 2) (Gururani, et al., 2015).

PSII photochemistry is driven by fast induction kinetics in which it contributes to crucial information regarding the photosystem II photochemistry and electron acceptor reduction in photosynthesis (Stirbet & Govindjee, 2011). By using specialized software and mathematical calculations, OJIP analyses present this information in an easy-to-understand manner. The slow fluorescence method has been used as a method for assessing plant performance under high salinity, as well as for detecting plant senescence. As slow fluorescence intensity changes with age and hormonal modulation of senescence, differences in photosynthesis capacity and chlorophyll content can be observed (Strasser & Govindjee, 1991).

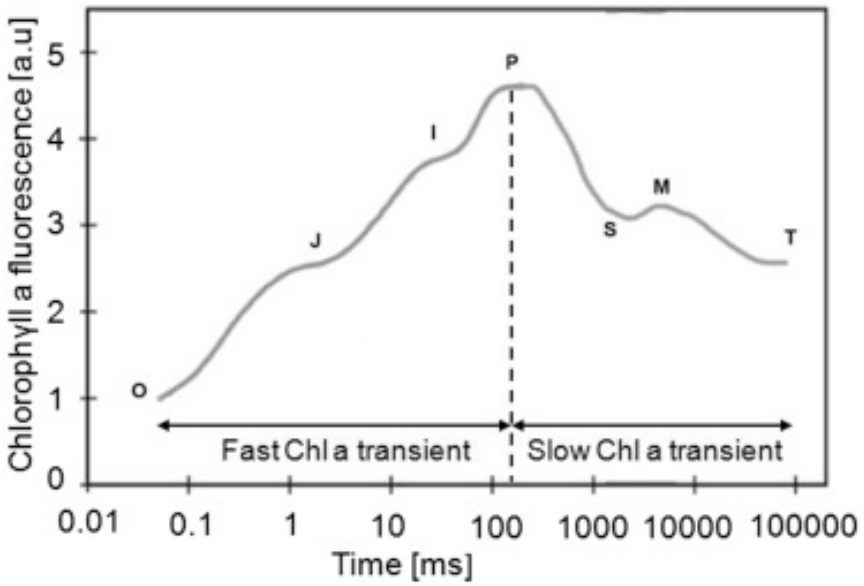

Figure 2: An Overview of the Chlorophyll Fluorescence Induction Curve in Plant Samples.

PSII efficiency can be determined using the fast chlorophyll transient kinetics, both under normal and stressful conditions. Because fast chlorophyll is analyzed during stress conditions, transients can be used to detect some details regarding adjustments and modifications in the photosynthetic machinery. PSII is affected by abiotic stress according to the parameter Fv/Fm. The reactivity of the photosynthetic apparatus determines the

physiology and vitality of plants exposed to environmental stresses (Gururani, 2022). As a technique for determining reactivity, measurements of fast chlorophyll a fluorescence transients have become quite common. An analysis by Swoczyna et al. (2022) of the effects of nutrition on photosynthetic apparatus vitality revealed chlorophyll a fluorescence techniques were useful in determining if there was a reduction in overall photosynthetic apparatus vitality.

1.3.2 Salt Tolerant Crops and Transgenic Plant Uses

Agriculture faces serious threats from abiotic stress. Due to global warming, these factors will become more prevalent in coming decades. Improved varieties of crop plants that can withstand a variety of stresses are needed in order to maintain the current rate of agricultural production growth. Developing stress-tolerant plants requires an understanding of how plants cope with adverse environments. Several species of plants have been studied for their stress tolerance mechanisms, including *Arabidopsis*. The specific mechanisms involved in a particular crop's stress tolerance must be investigated, as well as the accumulating information on stress tolerance mechanisms of plants, in order to develop stress-tolerant varieties of that crop. We must, therefore, improve our understanding of stress tolerance mechanisms and identify stress tolerance genes in agriculturally important plants, such as potatoes, tomatoes, rice, etc. (Amudha & Balasubramani, 2010).

A study of published literature from the past two decades suggests that salt-tolerant genetically engineered crops are the most effective strategy for mitigating salinity. In addition, as the water table drops, salt residues remain in the rhizosphere, so rhizosphere tolerance is not only required for dewatering species, but in addition also for the following annual crops. Furthermore, introducing salt tolerance to crops allows them to utilize poor-quality irrigation water more effectively. To determine the effect of salt on plant development, it is also important to understand the mechanisms of salt tolerance at the whole-plant, molecular, and organelle levels. Salt stress changes gene expression patterns as well as protein production. In addition, there are some disagreements whether salinity can activates genes related to salt stress, despite quantitative changes in protein synthesis caused by salinity.

Due to the lack of evidence that salt tolerance is conferred by any unique gene(s), a variety of genes are activated during abiotic stress, leading to a significant increase in osmoprotectants, secondary metabolites, hormones antioxidant enzymes and proteins that act as defenses against abiotic stress (Sharma & Gayen, 2021). In the absence of a complete understanding of how plants tolerate stress, the use of transgenic approaches to improve crop performance has not been entirely successful. According to Esmaeili et al. (2019), plant tolerance to salt, drought, and heat stress is enhanced significantly when *OsSIZ1* and *AVP1* genes are co-overexpressed in *A. thaliana*.

1.3.3 Auxin-Repressed Protein (ARP)

Higher plants have auxin-repressed proteins (*ARPs*), which control gene expression in order to promote growth and development. Some isoforms of this gene include dormancy-associated protein homolog 1 (*DRMH1*) and dormancy- associated protein 2 (*DRM2*) (Goudert et al., 2023). Several species of plants express ARP1 both in dormant buds and non-growing tissues (Souza et al., 2019). The growth-stimulating phytohormone auxin regulates a variety of plant growth and development processes, including vascular differentiation, apical dominance, lateral root formation, shoot elongation, and embryo patterning (Lee et al., 2013). Dormancy-related proteins and glycine-rich proteins are members of the *ARP* gene family. GERI/ARP1 silencing increased plant susceptibility to infection by Tobacco mosaic virus, *Pectobacterium carotovorum* subsp. carotovora, and *Phytophthora parasitica* var. Nicotianae (Souza et al., 2019). While this gene has been studied for its function in plant growth and development in addition to resistance to biotic stress, its role in abiotic stress tolerance is not well understood.

The putative auxin-repressed protein identified in a study conducted in *Capsicum annum* exhibited upregulation under cold and salt stress, suggesting its involvement in the cold and salt defense mechanisms (Hwang et al., 2005). Using a large-scale yeast functional screening approach, Gangadhar et al. (2014) identified potential drought tolerance genes in *Solanum tuberosum* (potato). The authors report that twenty genes are affected by drought, 14 by salinity, and 11 by heat/drought/salt stress. In this study, we have cloned into *Arabidopsis* one of these 20 genes, the auxin repressed protein (*ARP1*). In this proposed study, the functional characterization of ARP1 transgenic *Arabidopsis* lines was

performed under salinity stress conditions, employing detailed physiological, molecular, and biochemical approaches.

The study was designed to test the hypothesis that salt stress tolerance could be conferred to *Arabidopsis thaliana* by overexpression of the potato *ARP1* gene encoding Auxin-repressed protein. The following objectives were intended to be achieved by this study:

1. To determine the functional role of *ARP1* in plant growth, development, and abiotic stress.
2. To evaluate the performance of transgenic *Arabidopsis* plants expressing *ARP1* under salinity stress.
3. To study the effect of stress conditions on photosynthetic parameters in control and transgenic plants.
4. To determine the putative involvement of *ARP1* gene in stress-related hormone biosynthesis.

Chapter 2: Material and Methods

2.1 In slico Analysis of ARP1

A BLAST search was performed to identify potential orthologs using the solanum tubersisum ARP1 mRNA sequence (NCBI Accession: JX576266) to identify the species with the highest sequence similarity with StARP. Subsequently, the sequences were aligned using the ClustalW online program to construct a phylogenetic tree. Protein sequence was obtained from Uniprot (Uniprot ID: K7VPG9) and the three dimensional structure of protein was predicated using SwissModel, hosted at the Biozentrum, University of Basel.

2.2 Generation of Plant Material

The full-length coding region of the ARP1 gene (JX576266), which spans 384 base pairs, was amplified from a potato cDNA library using appropriate primers (Table 1). Subsequently, it was cloned into a plant expression vector, pMDC32, containing a CaMV 35S promoter for expression of the transgene in plants.

An *Agrobacterium tumefaciens* mediated transformation method (Clough and Bent, 1998) was employed to generate *A. thaliana* (Col-0) widely used reference strain of the model *Arabidopsis* plants over expressing the ARP1 gene. Putative transgenic plants were selected on half strength Murashige and Skoog medium (PT021, Himedia, India) amended with hygromycin and agar. Integration of transgene cassette into genomic DNA were verified using PCR amplification of *Hygromycin phosphotransferase* gene fragment using particular primers (Table 1). Further, two homozygous lines of transgenic plants, ARP1-L1 and ARP1-L2, overexpressing the ARP1 gene were selected for further experiments. Both the gene cloning and generation of transgenic plant were performed at Department of Molecular Biotechnology, Konkuk University, Seoul, South Korea.

Table 1: List of the primers used in the study.

Gene name	Accession	Primer	Primer Sequence (5' – 3')
*ARP1**	JX576266	StARP1_F	CACCATGGTGTTAATTGAA
		StARP1_R	TCACTGATGCTTGGATCGGGTAT
*Hygromycin phosphotransferase***	-	PMDCHyg_F	TTAGCGAGAGCCTGACCTATTGCATC
		PMDCHyg_F	CAGAAGAAGATGTTGGCGACCTCGTA
*ARP1***	JX576266	ARP1_F	TCTCTGTCTATGCCGGGCT
		ARP1_R	CACTGATGCTTGGATCGGGT
*Glyceraldehyde-3-phosphate dehydrogenase C2****	AT1G13440	GAPDH_F	GAGAGTTTGTGTGTGGTTGAGTTC
		GAPDH_R	GGTTTGAGTTAGCACGAGAAAGTAA
*Catalase****	AT1G20630	CAT_F	AAGTGCTTCATCGGGAAGGA
		CAT_R	CTTCAACAAAACGCTTCACGA
*Superoxide dismutase****	AT1G08830	SOD_F	TCCATGCAGACCCTGATGAC
		SOD_R	CCTGGAGACAATGATGCC
*Ascorbate peroxidase****	AT1G07890	APX_F	CCGTCCTTTGGTCGAGAAATA
		APX_R	GGATAAGTACCCAAGCTCAGAAA

*Used for gene isolation and cloning into the pMDC32 vector.

**Used for validation of transgenic plants as well as RT-qPCR.

***Used only for RT-qPCR.

2.3 Salinity Tolerance Test

Wild-type (WT) and transgenic ARP1-L1 *and* ARP1-L2 *A. thaliana plants* grown in controlled conditions were used for the salinity tolerance test. Initially, seeds of wild type and transgenic plants were immersed in sterile distilled water and kept in dark at 4°C for two days of cold treatment. The cold treated seeds were further sown on to seedling trays filled with potting mix (Van Egmond, Naarden, Netherlands) for germination in an illuminated growth chamber (16h light/8 h dark, temperature 23 ± 1°C). Three-week-old plants were watered with 200 mM NaCl solution to trays underneath the pots containing the plants. Normal tap water was given to WT non-stressed or transgenic plants. After three weeks of stress treatment, physiological measurements were taken, and samples were collected, 10-15 replicate to assess the difference in salt tolerance between wild-type and transgenic plants. The collected samples and data were labeled as WT-NS (WT no salt), WT-NaCl (WT NaCl treated), ARP1-L1/L2-NS (Transgenic no salt) and ARP1-L1/L2-NaCl (Transgenic salt treated).

2.4 Measurement of Growth Parameters

After three weeks of stress treatment, measurements were taken for the height of the plant, root length, number of leaves per plant and fresh weight of both control and treated plants.

2.5 Measurement of Stomatal Conductance

Stomatal conductance was measured on the adaxial surface of fully developed intact rosette leaves of plants using a steady-state diffusion leaf porometer (model SC-1; Decagon Devices Inc., Pullman, WA, USA). The porometer was calibrated prior to taking measurements, and stomatal conductance was assessed under normal laboratory lighting and temperature conditions.

2.6 Measurement of Electrolyte Leakage

Electrolyte leakage was assessed following the method developed by Sullivan and Ross (Sullivan & Ross, 1979). Leaf discs were placed in a boiling tube containing 10ml of deionized water, and the initial electrical conductivity (ECa) was measured. The tubes were then subjected to heating at 45°C and 55°C for 30 minutes each in a water bath,

after which the electrical conductivity (ECb) was measured over. Then, the tubes were heated at 100°C for 10 minutes to obtain the final electrical conductivity (ECc). The percentage of electrolyte leakage was calculated using the formula: Electrolyte leakage (%) = ECb – Eca / ECc x 100.

2.7 Estimation of Chlorophyll-a Fluorescence

Specific regions on fully expanded topmost rosette leaves of the *A. thaliana* plants were dark-adapted for 1h using a clip, following which chlorophyll-a (Chl a) fluorescence measurements were taken using the Pocket PEA (Hansatech Instruments Ltd., King's Lynn, UK). The collected data were then analyzed using the Biolyzer software program based on the "JIP-test equations," as outlined by Gururani et al. (2013). In summary, parameters that includes maximal fluorescence (FM) and minimal fluorescence (FO) of the sampled leaves were utilized to calculate the quantum yield of Photosystem II (PSII), expressed as the Fv/FM ratio. Furthermore, various other photosystem-related parameters, as described in previous studies (Alyammahi & Gururani, 2020; Varghese et al., 2019) were derived from the collected data.

Table 2: Formulae and glossary of terms used by the JIP-test for the analysis of Chl a fluorescence transient OJIP emitted by dark-adapted photosynthetic samples. (Modified after Strasser et al. 2004).

Data extracted from the recorded fluorescence transient OJIP	
F_t	fluorescence at time t after onset of actinic illumination
$F_{50\mu s}$ or $F_{20\mu s}$	minimal reliable recorded fluorescence, at 50 μs with the PEA- or 20 μs with the Handy-PEA-fluorimeter
$F_{300\mu s}$	fluorescence intensity at 300μs
$F_J \equiv F_{2ms}$	fluorescence intensity at the J-step (2 ms) of OJIP
$F_I \equiv F_{30ms}$	fluorescence intensity at the I-step (30 ms) of OJIP
F_P	maximal recorded fluorescence intensity, at the peak P of OJIP
t_{F_M}	time (in ms) to reach the maximal fluorescence intensity F_M
Area	total complementary area between the fluorescence induction curve and $F = F_M$
Fluorescence parameters derived from the extracted data	
$F_0 \cong F_{50\mu s}$ or $\cong F_{20\mu s}$	minimal fluorescence (all PSII RCs are assumed to be open)
F_M (= F_P)	maximal fluorescence, when all PSII RCs are closed (equal to F_P when the actinic light intensity is above 500 μmol photons m^{-2} s^{-1} and provided that all RCs are active as Q_A reducing)
$F_{\Delta t} \equiv F_t - F_0$	variable fluorescence at time t
$F_V \equiv F_M - F_0$	maximal variable fluorescence
$V_t \equiv F_{\Delta t}/F_V \equiv (F_t - F_0)/(F_M - F_0)$	relative variable fluorescence at time t
$M_0 \equiv [(\Delta F/\Delta t)_0]/(F_M - F_{50\mu s})$ $\equiv 4(F_{300\mu s}-F_{50\mu s})/(F_M - F_{50\mu s})$	approximated initial slope (in ms^{-1}) of the fluorescence transient normalised on the maximal variable fluorescence F_V
Specific energy fluxes (per Q_A-reducing PSII reaction center - RC)	
$ABS/RC = M_0 (1/V_J)(1/\varphi_{Po})$	absorption flux (of antenna Chls) per RC
$TR_0/RC = M_0 (1/V_J)$	trapped energy flux (leading to Q_A reduction) per RC
$ET_0/RC = M_0 (1/V_J)\psi_{Eo}$	electron transport flux (further than Q_A^-) per RC
$RE_0/RC = M_0 (1/V_J)\psi_{Eo}\,\delta_{Ro}$	electron flux reducing end electron acceptors at the PSI acceptor side, per RC
Quantum yields and efficiencies	
$\varphi_{Pt} \equiv TR_t/ABS = [1-(F_t/F_M)] = \Delta F_t/F_M$	quantum yield for primary photochemistry at any time t, according to the general equation of Paillotin (1976)
$\varphi_{Po} \equiv TR_0/ABS = [1-(F_0/F_M)]$	maximum quantum yield for primary photochemistry
$\psi_{Eo} \equiv ET_0/TR_0 = (1-V_J)$	efficiency/probability for electron transport (ET), i.e. efficiency/probability that an electron moves further than Q_A^-
$\varphi_{Eo} \equiv ET_0/ABS = [1-(F_0/F_M)]\psi_{Eo}$	quantum yield for electron transport (ET)
$\delta_{Ro} \equiv RE_0/ET_0 = (1-V_I)/(1-V_J)$	efficiency/probability with which an electron from the intersystem electron carriers moves to reduce end electron acceptors at the PSI acceptor side (RE)
$\varphi_{Ro} \equiv RE_0/ABS = [1-(F_0/F_M)]\psi_{Eo}\,\delta_{Ro}$	quantum yield for reduction of end electron acceptors at the PSI acceptor side (RE)
$\gamma_{RC} = Chl_{RC}/Chl_{total} = RC/(ABS+RC)$	probability that a PSII Chl molecule functions as RC
$RC/ABS \equiv \gamma_{RC}/(1-\gamma_{RC}) = \varphi_{Po}(V_J/M_0)$	Q_A-reducing RCs per PSII antenna Chl (reciprocal of ABS/RC)
Performance indexes (products of terms expressing partial potentials at steps of energy bifurcations)	
$PI_{ABS} \equiv \frac{\gamma_{RC}}{1-\gamma_{RC}} \cdot \frac{\varphi_{Po}}{1-\varphi_{Po}} \cdot \frac{\psi_o}{1-\psi_o}$	performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors
$PI_{total} \equiv PI_{ABS} \cdot \frac{\delta_{Ro}}{1-\delta_{Ro}}$	performance index (potential) for energy conservation from exciton to the reduction of PSI end acceptors

2.8 Leaf Spectrometer Measurements for Assessment of Stress

The spectral data obtained from leaves in response to stressors can serve as a instrument for quantifying plant stress tolerance. In this study, the topmost fully grown rosette leaves from each plant were harvested, and spectral readings were promptly taken using the CI-710s SpectraVue Leaf Spectrometer (CID Bio-Science, Washington, USA). Subsequently, the transmission, absorption, and reflection data recorded were analyzed to derive various indicators of plant health/stress levels (such as NDVI, PSRI, CRI1, WBI, NPCI, ARI1, FRI1, PRI, CCI, and greenness) and pigment content using built-in indices provided by the spectrometer.

2.9 Estimation of Malondialdehyde Content

Around 500 mg of leaf samples were crushed to a powder using the liquid nitrogen and then smoothed in 5 mL of 50 mM buffer solution (comprising 0.07% $NaH_2PO_4 \cdot 2H_2O$ and 1.6% $Na_2HPO_4 \cdot 12H_2O$). Following homogenization, the mixture was centrifuged at 20,000 g for 25 minutes at 4°C. Subsequently, 4 mL of 20% trichloroacetic acid containing 0.5% thiobarbituric acid were added to 1 mL of the resulting supernatant. The mixture was then incubated at 95°C for 30 min, cooled on ice, and centrifuged at 10,000 g for 10 min, and the absorbance of the supernatant was measured at 532 nm and 600 nm. The absorbance value at 600 nm was subtracted from the absorbance reading at 532 nm to correct for any non-specific absorption. The concentration of malondialdehyde (MDA) was then determined using an MDA extinction coefficient of 155 mM–1cm–1, as described by Fu et al. (2001).

2.10 Determination Chlorophyll Content

Chrorophyll content was determined by grinding leaf samples in 2 mL of 80% acetone and held overnight at 4°C. Following the dark incubation, the mixture was centrifugated at 3000 g for 5 min and the supernatant absorbance was recorded at 663, 645, and 652 nm and chlorophyll content were determined as described earlier by Arnon (1949).

2.11 Proline Estimation

Proline content in the leaves was estimated using a colorimetric method previously outlined (Gururani et al., 2013). Initially, around 500 mg of leaf samples were crushed into

a fine powder using liquid nitrogen, followed by homogenization in 10 mL of 3% aqueous sulfosalicylic acid. Subsequently, equivalent volumes (2 mL each) of the filtered homogenate, acid-ninhydrin, and glacial acetic acid were combined and allowed to incubate at room temperature. After 1h, the reaction was halted by cooling the tubes on ice. The chromophore-containing phase was then extracted with 4 mL of toluene, and its absorbance was measured at 520 nm. Finally, the proline concentration in the samples was determined by plotting the absorbance values against a standard curve constructed using known concentrations of proline.

2.12 Estimation of ROS Enzyme Activity

Leaf samples were crushed in a chilled mortar-pestle in the presence of liquid N2. Total protein was extracted with a buffer containing 0.2 M potassium phosphate buffer (pH 7.5), 50% (v/v) glycerol, 16 mM $MgSO_4$, 0.2 mM phenyl methyl sulfonyl fluoride, and 0.2% polyvinylpolypyrrolidone and centrifuged at 13,000 × g for 30 min at 4°C. The supernatant was collected, and the protein content was determined, as described earlier (Bradford, 1976). Specific enzyme activities for ascorbate peroxidase (APx), catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR) were determined following the methods described earlier (Elavarthi & Martin, 2010; Gururani et al., 2013).

2.13 Quantitative PCR Analysis of Genes Related to ROS Scavenging, Photosynthesis, and Salinity Response

Total RNA was isolated from different samples using ISOLATE II RNA Plant Kit (Bioline, BIO-52077). Five hundred nano gram of total RNA per sample was used for cDNA synthesis using SensiFAST cDNA Synthesis Kit (Bioline, BIO-65053). The cDNA was diluted to 1:4 ration using nuclease free water. Five microliters of diluted cDNA was used as a template for the qRT-PCR. The qRT-PCR reaction was run on QuantStudio™ 5 Real-Time PCR System (A34322, Thermo Fisher) using SensiFAST SYBR Lo-ROX Kit (BIO-94005, Bioline, UK) master mix. Glyceraldehyde-3-phosphate dehydrogenase gene (AT1G13440) was used as a housekeeping gene. Expression of different genes related to ROS scavenging, photosynthesis, and salinity response listed in Table 1 were determined. Relative transcript level genes were calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen &

Livak, 2008). Mean values of relative gene expression were calculated based on data collected from four biological replicates.

2.14 Statistical Analyses

All experiments were conducted using completely randomized block design. Experiments were repeated at least three times with fifteen to twenty replicates. Statistical analyses were performed using Origin 8.0 software program.

Chapter 3: Results

3.1 *In Silico* Analyses of ARP Gene

According to the multiple sequence alignment of the mRNA outcomes revealed that ARP is a preserved protein with high sequence similarity with dicots of Brassicaceae, and Solanaceae families as well as monocot grasses like rice, and maize (Figure 3). BLAST analysis of Auxin Repressed Protein1 (*ARP1*) from *S. tuberosum* and other plant species (Table 3), based on these result, they have high percentage identity with Solanum verrucosum. Protein sequence was obtained from uniprot (K7VPG9) and the modelling was done in Swiss model. Modelling showed a sequence identity of 70% with A0A803LLK4.1.A (Auxin-repressed protein AlphaFold DB model of A0A803LLK4_CHEQI (gene: A0A803LLK4_CHEQI, organism: *Chenopodium quinoa* (Quinoa)) (Figure 4). Furthermore, subcellular localization study revealed that ARP gene is expressed primarily in the cytoplasm (Figure 5).

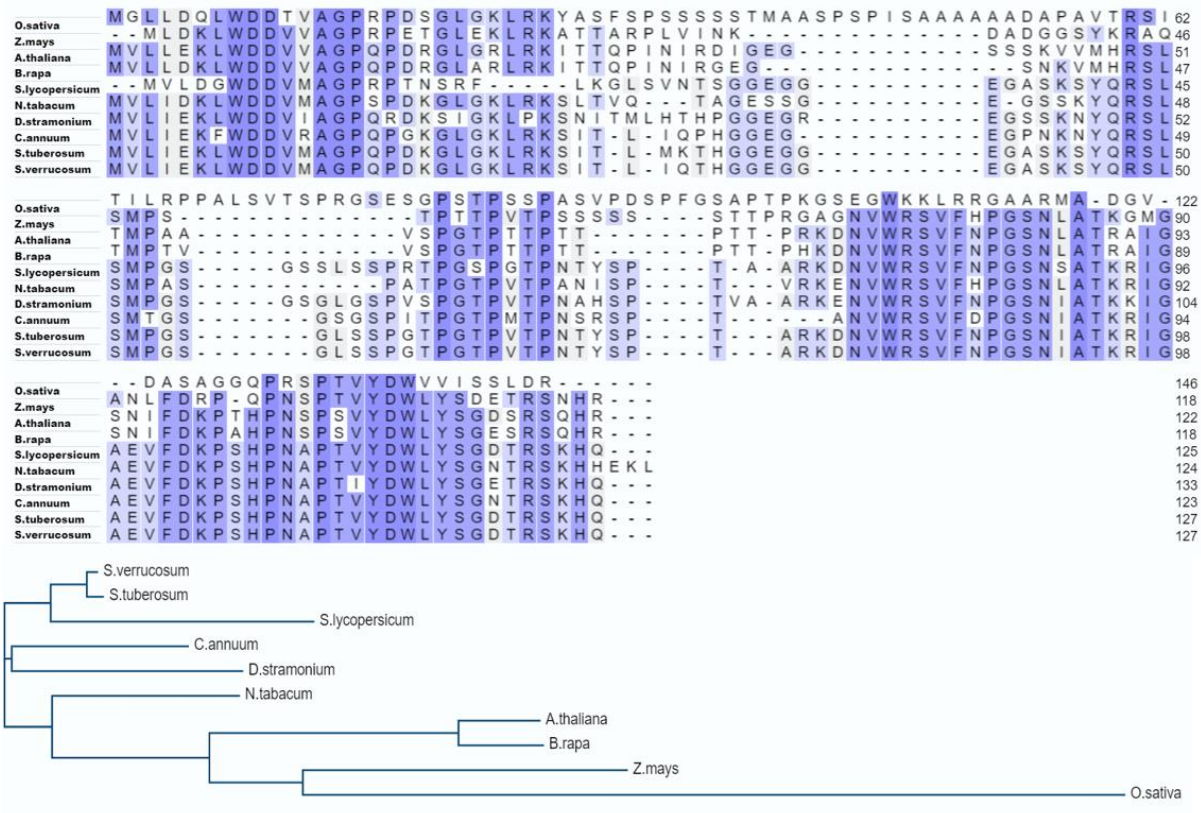

Figure 3: Sequence alignment and phylogenetic analysis of Auxin Repressed Protein (ARP) from S. tuberosum and other plant species.

Table 3: BLAST analysis of Auxin Repressed Protein1 (ARP1) from S. tuberosum and other plant species.

Scientific name	Max Score	Query Cover (%)	E value	Per. Ident (%)	Accession
Solanum tuberosum	260	100	3.00E-90	100	AFW90622.1
Solanum verrucosum	258	100	4.00E-89	98.43	XP_049361215.1
Solanum lycopersicum	214	100	1.00E-71	89.23	XP_004234112.1
Capsicum annuum	108	43	7.00E-31	87.27	AAR83888.1
Datura stramonium	108	43	1.00E-30	87.27	MCE3052407.1
Nicotiana tabacum	197	100	4.00E-65	78.74	AAS76635.1
Zea mays	75.9	37	7.00E-18	70.83	ONM05034.1
Oryza sativa	46.2	20	9.00E-06	69.23	KAB8091742.1
Arabidopsis thaliana	146	100	6.00E-45	62.79	NP_001154378.1
Brassica rapa	137	100	9.00E-42	59.84	XP_009113675.2

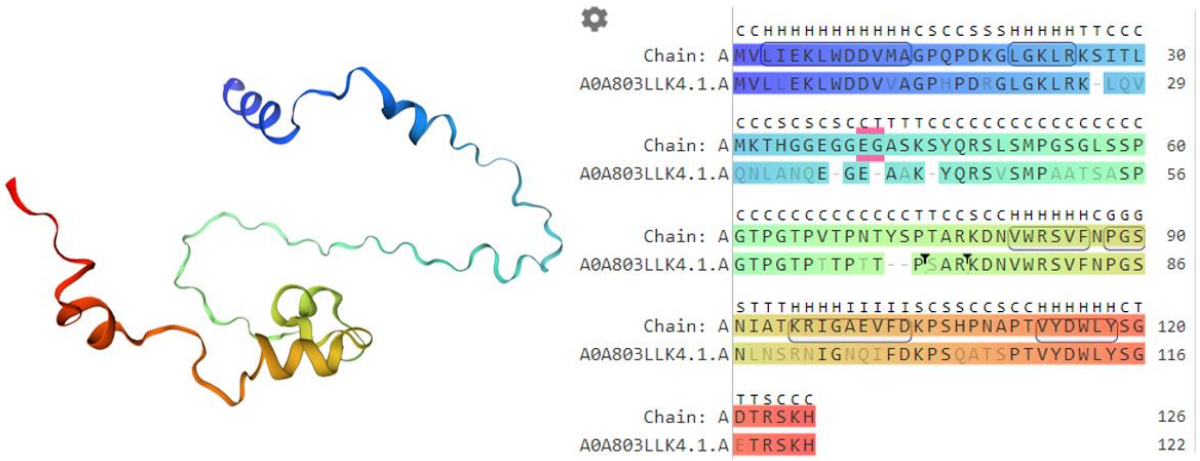

Figure 4: Protein modelling of potato Auxin Repressed Protein1 (ARP). Performed using SwissModel, Biozentrum, University of Basel.

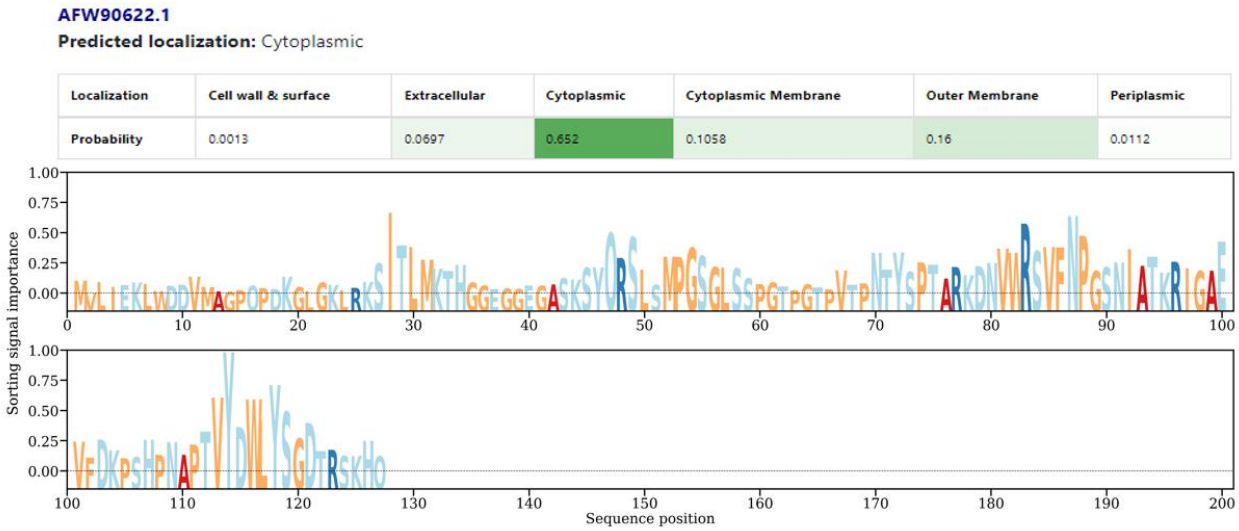

Figure 5: Subcellular localization of Auxin Repressed Protein1. (ARP1; NCBI ID: AFW90622.1) was predicted using DeepLocPro program, Denmark Technological University.

3.2 Transgenic Line Selection

In Figure 6A, pMDC32-*StARP* construct was used to generate ARP transgenic *A. thaliana* plants. PCR was used for the selection of the transgenic gene using the Hygromycin phosphotransferase gene (457 bp) from genomic DNA. L1 and L2 corresponds to ARP1 and ARP2, L3 and L4 corresponds to positive and negative control plants and L5 is DNA ladder (Figure 6B). Relative expression of *StARP* gene in Arabidopsis wild-type (Col) and transgenic ARP plants to the primer GAPDH. The ARP1 and ARP2 transgenic lines looked notably greener and healthier than the WT plants (Figure 6D), demonstrating the advantageous role ARP protein plays in reducing salt toxicity in transgenic Arabidopsis plants. This difference was evident between the control and ARP1 and ARP2 lines treated with salt.

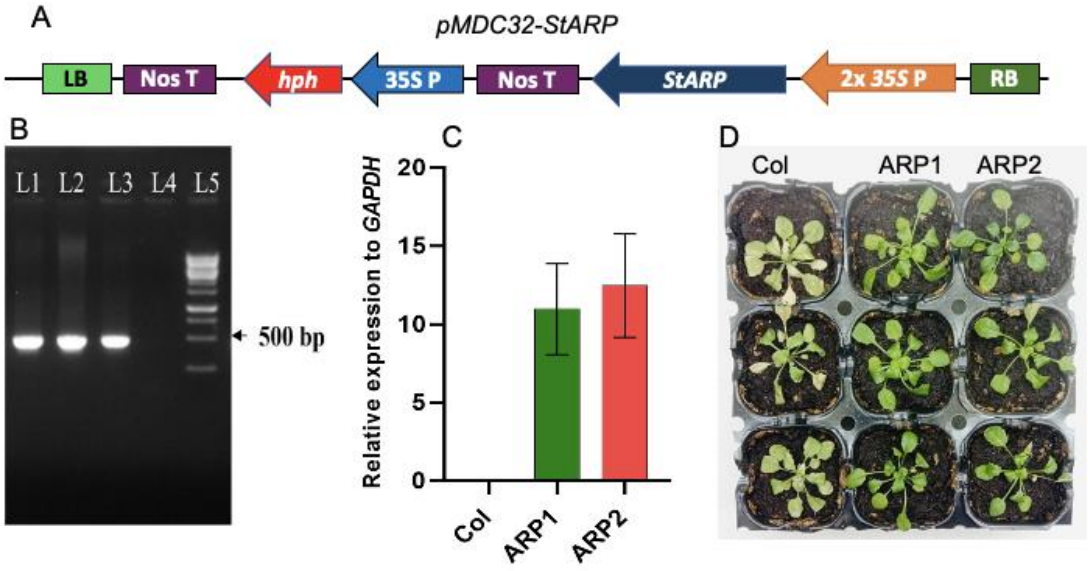

Figure 6: Transgenic line selection. A. pMDC32-StARP construct used to generate ARP transgenic A. thaliana plants. B. PCR amplification of Hygromycin phosphotransferase gene (457 bp) from genomic DNA of two hygromycin-resistant transgenics lines. C. Relative expression of *StARP* gene in Arabidopsis wild-type (Col) and transgenic ARP plants. D. Morphological differences between Arabidopsis wild-type (Col) and transgenic ARP plants after 10 days of exposure to 200 mM NaCl stress.

3.3 Molecular Analysis of Arabidopsis Plants Expressing Potato ARP Gene

PCR was used for the transgenic *A. thaliana* plants expressing potato ARP gene for confirmation and two lines (ARP1 and ARP2) were thus selected for functional characterization. As shown in Figure 7 potato ARP specific gene primers revealed no amplification in WT but were amplified in two ARP lines (ARP1 and ARP2) validating the integration of ARP in Arabidopsis.

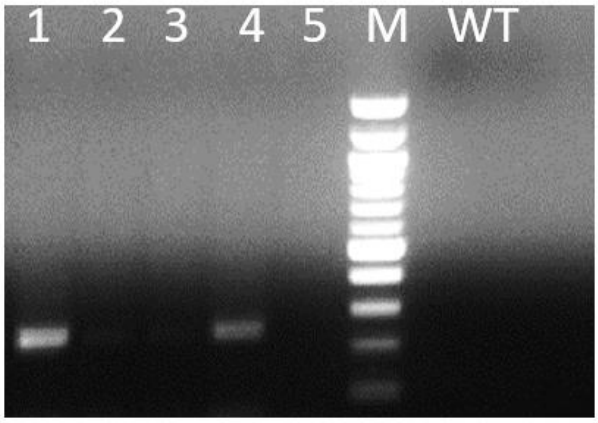

Figure 7: PCR confirmation of ARP1 (lane 1) and ARP2 (lane 4) transgenic Arabidopsis lines. Lanes 2, 3 and 5 show PCR negative putative transgenic Arabidopsis lines.

3.4 Evaluation of Transgenic A. Thaliana Lines Under Salinity Stress

Col-WW and ARP1 and ARP2 transgenic lines exposed to salinity stress (200 mM) showed a significant increase in stress tolerance in ARP1 and ARP2 transgenic lines when compared with Col (control). In present study revealed that, salinity decreased plant height in Col-NaCl and transgenic ARP1-NaCl and ARP2-NaCl by 26.66%, 19.23%, and 20% respectively; root lengths in Col-NaCl and transgenic ARP1-NaCl and ARP2-NaCl by 48.04%, 28.09%, and 30% respectively; and fresh weight in Col-NaCl and transgenic

ARP1-NaCl and ARP2-NaCl by 35.67%, 23.09%, and 23.18% respectively; compared with the respective values in Col-WW and ARP1-WW and ARP2-WW plants (Figure 8).

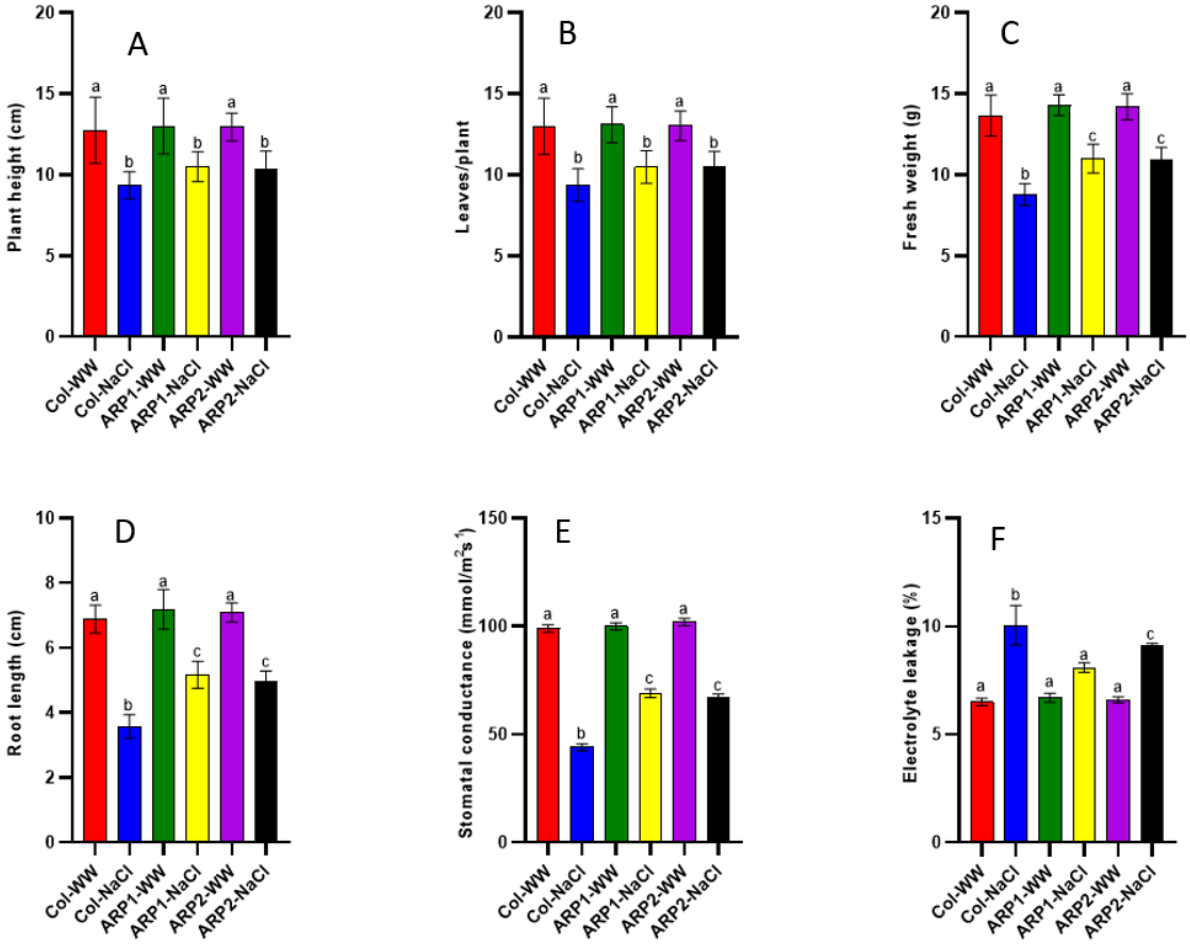

Figure 8: Morphological and physiological analysis of wild-type (Col) and transgenic ARP Arabidopsis line. (A) plant height, (B) leaves/plant, (C) fresh weight, (D) root length, (E) stomatal conductance, (F) electrolyte leakage in wild-type (Col) and transgenic ARP *Arabidopsis thaliana* plants under non-stress (Col-WW and ARP-WW) and salinity (Col-NaCl and ARP-NaCl) conditions. Different letters above bars indicate significant differences ($p \leq 0.05$) between plant types under non-stress and stress conditions using a Tukey's honestly significant difference test (n=5). Bars represent the means ± standard deviation.

3.5 The Leaf Spectral Analysis in WT And ARP Transgenic Lines Under Salt Stress

Plant stress has been linked to the spectral vegetation indices. These parameters can be divided into three categories: the water, greenness, and xanthophyll indices. Leaf spectral indices, including the normalized difference vegetation index (NDVI), carotenoid reflectance index 1 (CRI1), plant senescence reflectance index (PSRI), water band index (WBI), anthocyanin reflectance index 1 (ARI1), normalized pigment chlorophyll index (NPCI), flavanols reflectance index 1 (FRI1), photochemical reflectance index (PRI), chlorophyll content index (CCI), and greenness, all served to further illustrate the physiological states of the WT and ARP transgenic lines. However, salinity decreased NDVI content in Col-NaCl and transgenic ARP1-NaCl and ARP2-NaCl by 27.1 % and 19.5%, and 20.6% respectively, NPCI by 35.8% and 21.6%, and 21.9%, respectively, and PRI values by 30% and 22%, and 22.4%, respectively, related with the respective values in Col-WW and ARP1-WW and ARP2-WW plants (Figure 9A-J). Moreover, salt stress also decreased PSRI in Col-NaCl and transgenic ARP1-NaCl and ARP2-NaCl by 26.2% and 18.3%, and 19.1% respectively, ARI 1 by 28.5% and 21.6%, and 20.4%, respectively, CCI values by 29.2% and 21%, and 20.1%, respectively, and WBI values by 30.1% and 22.1%, and 22.5%, correspondingly related with the respective values in Col-WW and ARP1-WW and ARP2-WW plants. The results suggest that both transgenic ARP lines accumulated more photosynthetic pigments and had enhanced photosynthetic activity when exposed to salinity stress.

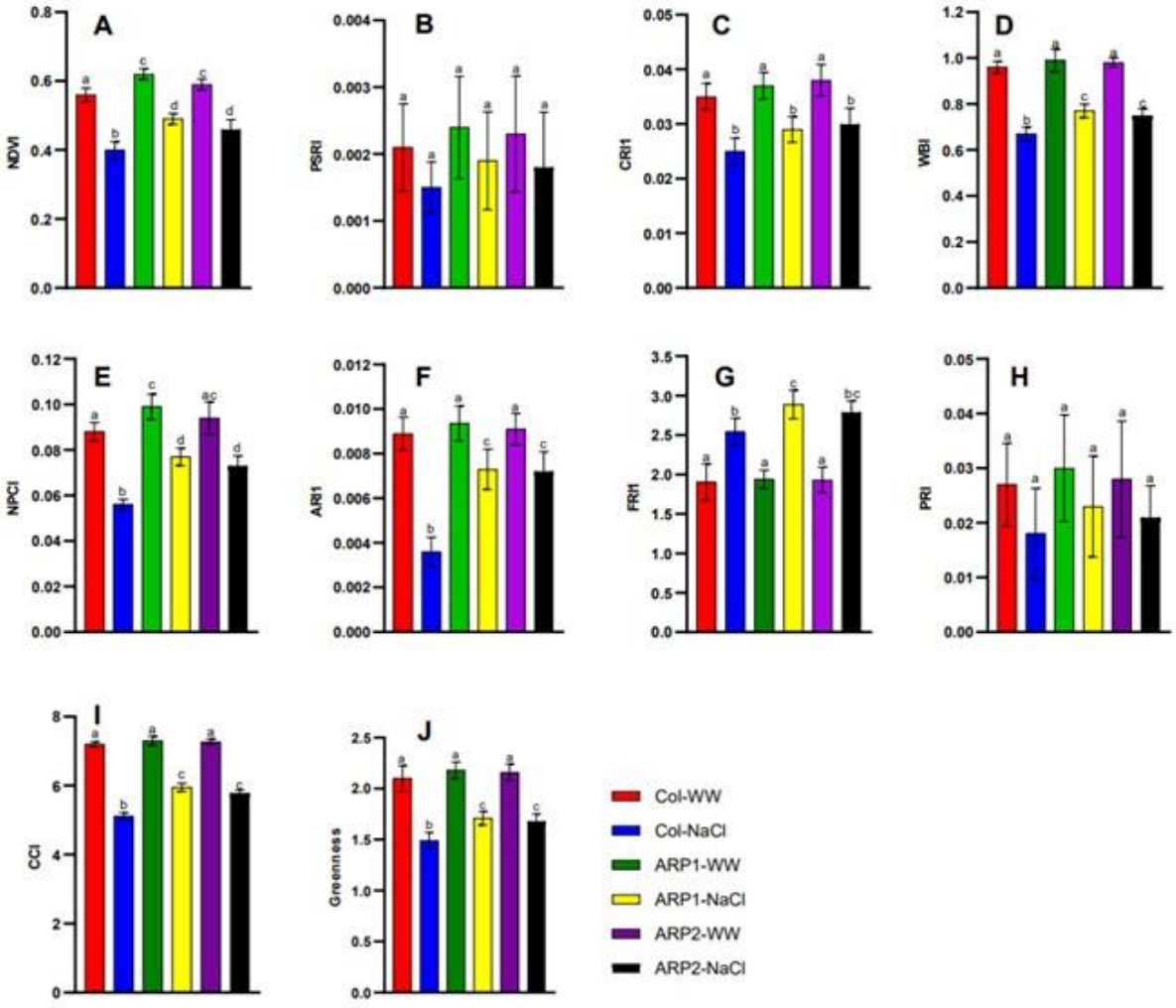

Figure 9: Leaf spectral analysis in wildtype (Col) and transgenic ARP Arabidopsis line. (A) NDVI, (B) PSRI, (C) CRI1, (D) WBI, (E) NPCI, (F) ARI1, (G) FRI1, (H) PRI, (I) CCI, and (J) Greenness in wild-type (Col) and transgenic ARP Arabidopsis thaliana plants under non-stress (Col-WW and ARP-WW) and salinity (Col-NaCl and ARP-NaCl) conditions. Different letters above bars indicate significant differences ($p \leq 0.05$) between plant types under non-stress and stress conditions using a Tukey's honestly significant difference test (n=5). Bars represent the means ± standard deviation.

3.6 Evaluation of Photosynthetic Pigment And PSII Efficacy In Salinized WT And ARP Arabidopsis Transgenic Lines

The measurement of chlorophyll in transgenic lines of Col and ARP further demonstrated the physiological differences between these plants. Following salt-induced stress, there was a discernible change between the content of the chlorophyll of the Col and ARP lines (Figure 10). Salinity decreased Chl a content in Col-NaCl and transgenic ARP1-NaCl and ARP2-NaCl by 35.86%, 21.62%, and 21.84% respectively, Chl b contents by 36.88%, 21.95%, and 24.79%, respectively, and total Chl contents by 36.16%, 21.90%, and 22.70%, respectively, compared with the respective values in Col-WW and ARP1-WW and ARP2-WW plants (Figure 10). Chl a, Chl b, and total Chl concentrations in the leaves of ARP-NaCl plants were higher than in the leaves of Col-NaCl plants, despite the fact that salinity had a detrimental effect on the photosynthetic pigments of both Col and transgenic ARP1 and ARP2 plants. These results suggest that under salinity-induced oxidative stress, transgenic ARP plants accumulated more photosynthetic pigments and hence had higher photosynthetic activity.

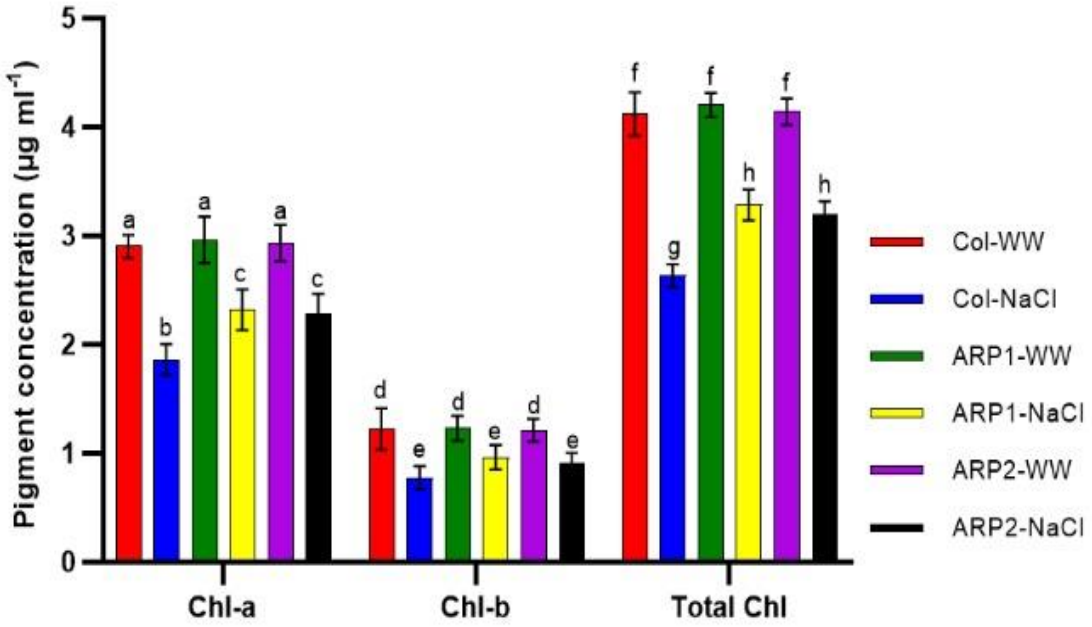

Figure 10: Chlorophyll estimation in wild-type (Col) and transgenic ARP Arabidopsis thaliana plants under non-stress (Col-WW and ARP-WW) and salinity (Col-NaCl and ARP-NaCl) conditions. Different letters above bars indicate significant differences ($p \leq 0.05$) between plant types under non-stress and stress conditions using a Tukey's honestly significant difference test (n=5). Bars represent the means ± standard deviation.

We used OJIP-test parameters (Table 4; Figures 11-13), which are complex markers of PSII efficiency under stress and plant photosynthetic ability, to quantify the kinetics of Chl a fluorescence. Figure 11 shows the typical fluorescence transient curves of WT and ARP plants under both condition normal and stress. Based on the result, Fluorescence curves indicated that the plants were variably photosynthetically active. A declining curve in Col-NaCl lines indicated that these WT plants suffered the most severe damage to its PSII components under salinity stress. The radar plot (Figure 12) further revealed that the damage to the PSII components was most severe in Col-NaCl group plants as indicated by the various PSII biophysical parameters studied. A phenomenological energy flux diagram, including ABS/RC, TRo/RC, ETo/RC, and DIo/RC, is shown in Figure 13. The fluxes provide insight into how active reaction centres (RCs) respond to light. In particular, ABS/RC indicates a rise in the number of active RCs, ETo/RC represents electron transport flux per reaction centre RCs, and TRo/RC shows trapped electron flux per reaction centre. Additionally, DIo/RC reveals the total energy dissipated per reaction center. Each parameter's magnitude is represented by the varying widths of the arrows. These parameters clearly demonstrated that ARP lines suffered significantly less damage under salinity stress than WT plants. Based on the analyses, we can observe that the overexpression of the ARP gene under salinity stress has improved both the photochemistry of photosystem II and the performance indices. Salinity reduced the energy absorption-based performance index (PIABS) by 79%, the maximum quantum yield for primary photochemistry (φPo) by 33.69%, and the quantum yield for electron transport (φEo) by 30.76% when comparing Col-NaCl plants to Col-WW plants. Furthermore, we found that under non-stress and salinity circumstances, ARP line expression in *A. thaliana* sustained the Fv/Fm of PSII; however, the Fv/Fm ratio in Col-NaCl plants dropped by 70.83% in comparison to Col-WW plants (Table 4). On the other hand, transgenic ARP1-NaCl plants showed no discernible increase in comparison to ARP1-WW plants, although specific energy flux, comprising total energy dissipated per reaction center (DIo/RC), showed a 3.5-fold increase in Col-NaCl plants compared to Col-WW plants. The aforementioned findings agreed with the stomatal apparatus performance values that were noted. Transgenic ARP1-NaCl plants had 56.81% greater stomatal conductance than Col-NaCl plants (Figure 8E). Altogether, the stomatal conductance

values and the kinetics of chlorophyll a fluorescence suggested that salinity had a deleterious effect on PSII, which in turn prevented electron transport at PS II's donor site in Col-NaCl plants. On the other hand, ARP1-NaCl transgenic plants showed increased photosynthetic capacity and improved PSII efficiency by inhibiting PSII damage caused by salinity.

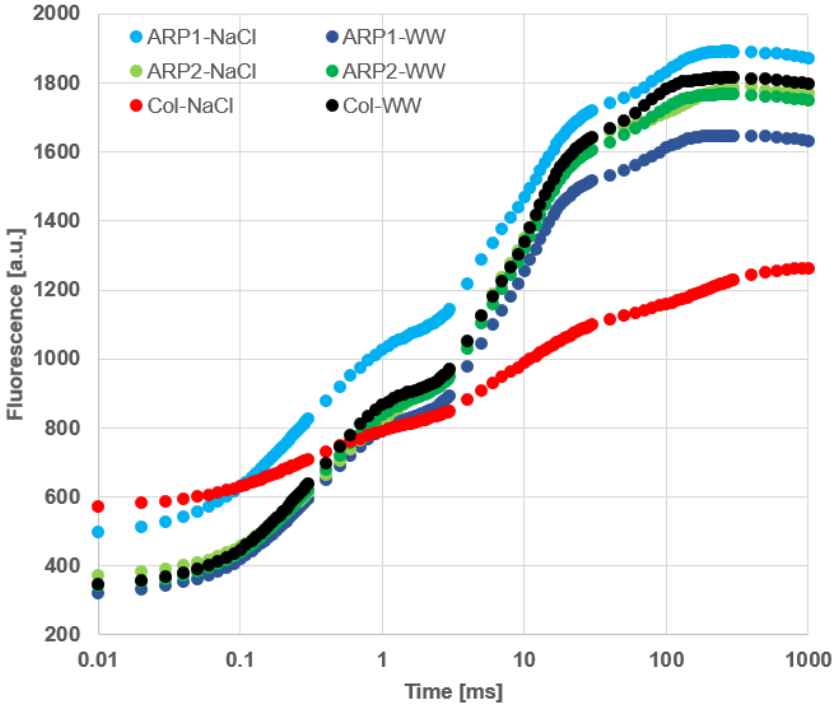

Figure 11: Fast chlorophyll A fluorescence kinetics (OJIP) in dark-adapted Arabidopsis leaves from the six experimental groups. The transient polyphasic curves for each line represent the average of 21 measurements, obtained from three replicates, each containing seven plants with respective groups.

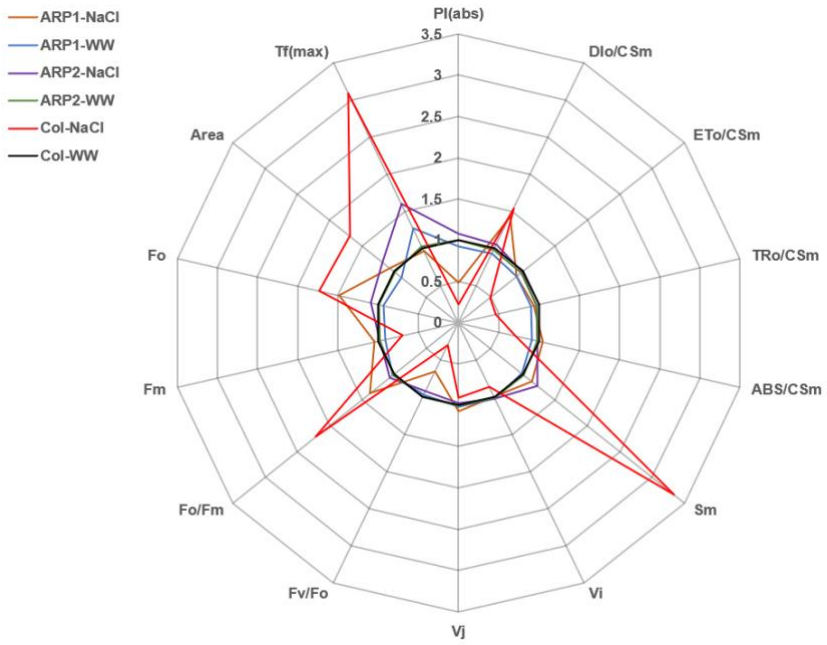

Figure 12: Radar plot showing various biophysical parameters of PSII. The details of the parameters studied are shown in Table 4. The values of each parameter for each line represent the average of 21 measurements, obtained from three replicates, each containing seven plants with respective groups.

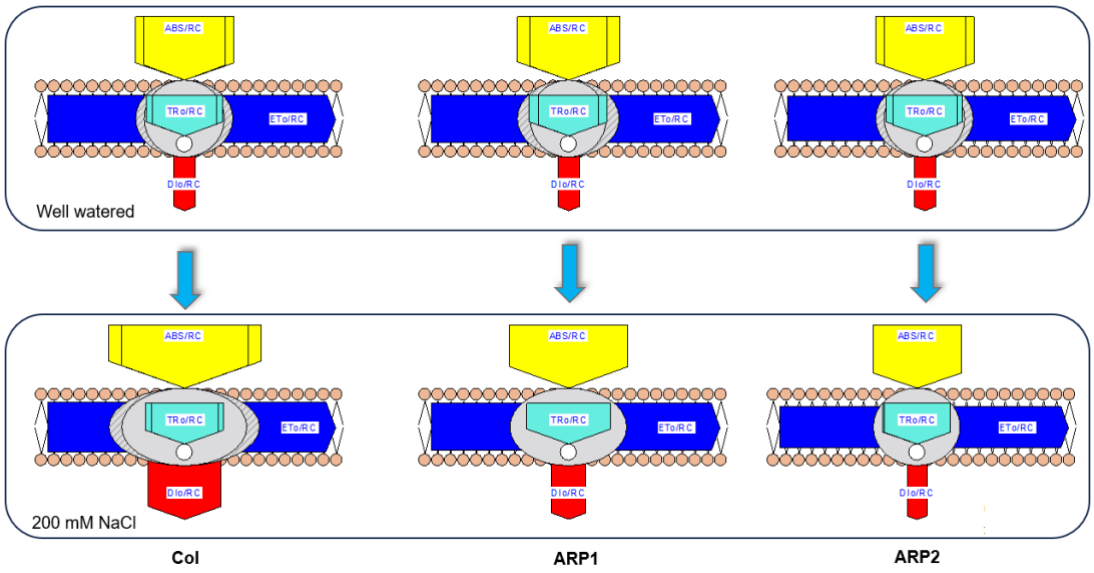

Figure 13: The energy pipeline leaf model of phenomenological fluxes (per reaction center; RC) in six groups. The relative changes in arrow width depict the value of each parameter. The values of each parameter for each line represent the average of 21 measurement, obtained from three replicates, each containing seven plants with respective groups.

Table 4: Effect of salinity on chlorophyll a florescence kinetics in wild-type (Col) and transgenic ARP Arabidopsis thaliana plants.

	ARP1-NaCl	ARP1-WW	Col-NaCl	Col-WW
PI(abs)	0.4883	0.9204	0.2274	1.0864
DIo/RC	1.632	1.0578	3.3899	0.9454
TRo/RC	1.0685	1.0247	1.0214	0.9758
ABS/RC	1.1898	1.0319	1.5314	0.9691
PHI(Do)	1.3716	1.0251	2.2137	0.9755
PHI(Eo)	0.8577	0.9858	0.702	1.0144
PSIo	0.955	0.9927	1.0526	1.0074
PHI(Po)	0.8981	0.9931	0.667	1.0069
Fv/Fo	0.6548	0.9686	0.3013	1.0324
Fo/Fm	1.3716	1.0251	2.2137	0.9755

3.7 Evaluation of Biochemical Parameters

The MDA content, a measure of lipid peroxidation, in Col-NaCl plants by 39.72% compared to Col-WW plants, and in ARP1-NaCl plants by 55.94% compared to ARP1-WW plants (Figure 14A). Furthermore, compared to ARP1-WW plants, the percentage of electrolyte leakage in Col-NaCl was only 20% higher, but it was 54.76% greater in Col-NaCl than in Col-WW plants (Figure 8F). Plant adaptation to stress can be positively indicated by proline content. The accumulation of proline demonstrated an increase in response to salt stress compared to the control plants (Figure 14B). We observed that salinity induced 43.30%, 56.14% and 54.54% increase in proline levels in Col-NaCl, ARP1-NaCl and ARP2-NaCl transgenic lines, compared with the respective values in Col-WW and ARP1-WW and ARP2-WW plants (Figure 14B). These findings suggested that

transgenic plants under salinity exhibited superior membrane integrity and osmotic adjustment than Col plants due to the ectopic expression of the ARP gene in *A. thaliana*.

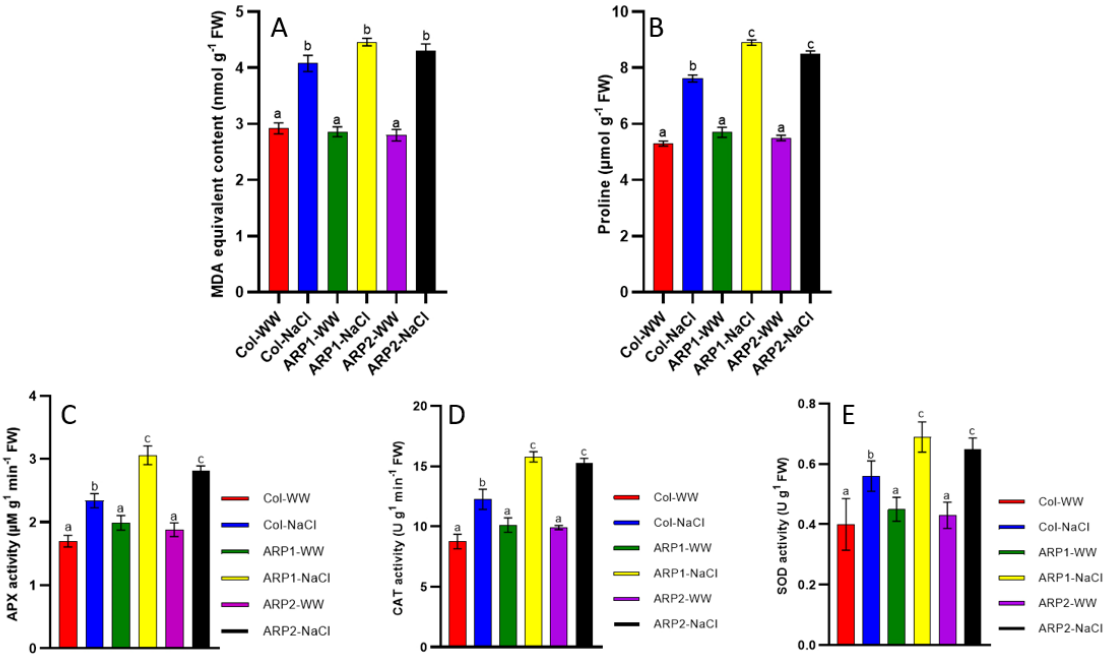

Figure 14: Biochemical analysis in wildtype (col) and transgenic ARP Arabidopsis line. (A) malondialdehyde content, (B) proline, (C) APX activity, (D) CAT activity and (E) SOD activity in wild-type (Col) and transgenic ARP Arabidopsis thaliana plants under non-stress (Col-WW and ARP-WW) and salinity (Col-NaCl and ARP-NaCl) conditions. Different letters above bars indicate significant differences ($p \leq 0.05$) between plant types under non-stress and stress conditions using a Tukey's honestly significant difference test (n=5). Bars represent the means ± standard deviation.

3.8 Analysis of Antioxidant Enzyme Activities and Gene Expression in Transgenic Lines of ARP

In plants that were exposed to salt, ARP1-NaCl plants showed greater activity of antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX), (Figure 13C-E). APX, SOD, and CAT are major enzymes in the ROS scavenging system. When transgenic ARP1 and ARP2 lines treated with salt stress had significantly higher APX activities when compared with WT plants (Figure 14C). In ARP1-NaCl plants compared to ARP1-WW plants, the enzyme activities of the APX and CAT genes were 81.81% and 75.1% higher, respectively (Figure 14C-D). These enzyme activity analyses were further confirmed with the expression analysis of genes encoding these antioxidant enzymes. ARP lines showed significantly higher expression of APX, SOD and CAT under salinity stress conditions compared to WT plants (Figure 15). This suggests that ARP's ectopic expression confers a greater antioxidant capacity and provides additional evidence of ARP's ability to enhance ROS-scavenging capacity in transgenic ARP plants exposed to salinity.

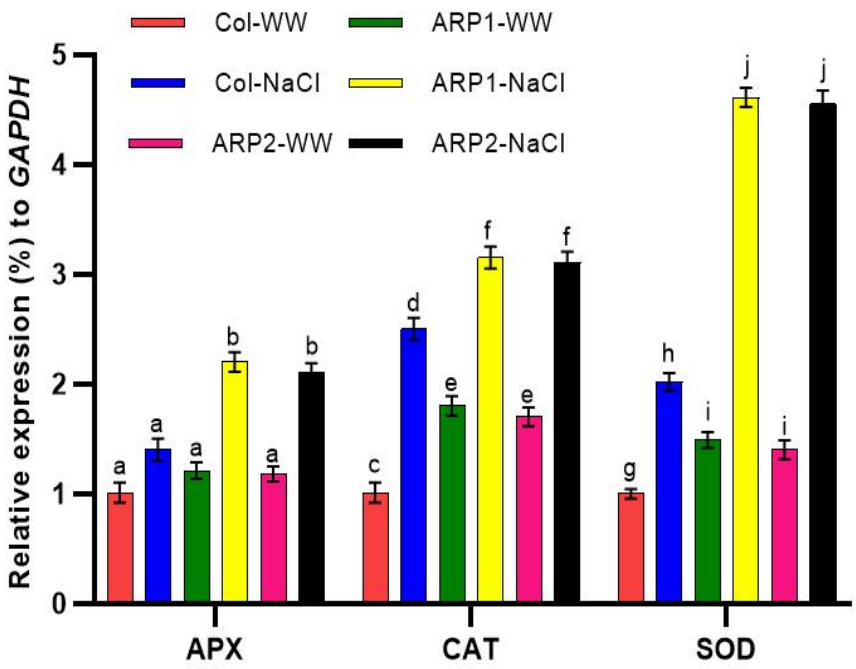

Figure 15: Expression of three antioxidant enzyme-encoding genes in wild-type (Col) and transgenic Arabidopsis thaliana ARP plants under non-stress (Col-WW and ARP-WW) and salinity (Col-NaCl and ARP-NaCl) conditions. Different letters above bars indicate significant differences ($p \leq 0.05$) between plant types under non-stress and stress conditions using a Tukey's honestly significant difference test (n=5). Bars represent the means ± standard deviation.

Chapter 4: Discussion

Soil salinization, stemming from global climate shifts and improper agricultural practices (Stavi et al., 2021), poses a significant challenge to plant cultivation. It's well-documented that salinization hampers the movement of essential nutrients and water in the soil (Shrivastava & Kumar, 2015). The osmotic balance and electrical gradient were disrupted due to the extreme uptake of Na^+ under high-salinity conditions, leading to disruptions in various physiological processes (Gill & Tuteja, 2010). Furthermore, the production of ROS during salinization results in oxidative harm to membranes and cell demise (Mirfattahi & Eshghi, 2023). Ultimately, the inhibition of photosynthetic processes (Nath et al., 2013) and the reduction in plant growth and productivity are detrimental consequences of salinization. Nevertheless, the capacity to transfer genes between different genetic reservoirs or modify the expression levels of native genes within a plant using genetic engineering offers a powerful approach to explore gene functionality and develop plants with enhanced stress tolerance (Kappachery et al., 2021). For example, compared to wild-type rice, transgenic plants that overexpress the ribosomal protein large subunit 6 (RPL6) under the control of the CaMV 35S promoter demonstrated improved salt tolerance and significantly increased grain output (Moin et al., 2021). Conversely, reducing the expression of the *GhRPL14B* gene negatively affected cotton (*Gossypium hirsutum)* seedling performance under salinity stress, as indicated by significant declines in various morphological and physiological traits (Shiraku et al., 2021). In a prior investigation, a yeast screening method identified 69 potential genes associated with drought stress in potatoes (Kappachery et al., 2013). The effectiveness of the potato StD26 gene in granting salinity tolerance and enhancing the survival rates of yeast transformants was highlighted by further evaluation of the transformants' relative tolerance to different abiotic stimuli, including salinity (Kappachery et al., 2013). Moreover, through various sequence alignment, conserved motifs in the ARP protein were revealed, indicating its substantial sequence similarity with proteins from various monocot and dicot species (Fig. 4). The presence of C2-C2 motifs in the ARP protein suggests its potential involvement in plant responses to abiotic stresses (Sun et al., 2021), aligning with the known roles of plant C2-C2 domain-containing proteins across various biological processes as noted by Corbalan-Garcia et al. (2003). Therefore, in order to clarify the function of the ARP gene

in salt tolerance, we went ahead and cloned and functionally characterized it in transgenic *Arabidopsis thaliana*. I compared the growth, biochemical, and physiological reactions of transgenic Arabidopsis ARP plants to control plants after they were exposed to 200 mM NaCl. The observation of healthier, greener leaves in ARP1-NaCl plants compared to the pale-greenish leaves of Col-WW plants indicated the superior growth performance of transgenic ARP1-NaCl plants under salinity stress (Figure 6D). Additionally, ARP1-NaCl plants exhibited longer root length compared to ARP1-WW plants (Figure 7D), suggesting that ectopic expression of the ARP gene enabled transgenic Arabidopsis to better cope with salinity stress in terms of growth. It is possible to explain the transgenic ARP1-NaCl plants' improved growth performance by their ability to sustain photosynthetic pigment levels and improve photosynthesis in the face of salt stress. Indeed, ARP1-NaCl plants exhibited higher levels of total chlorophyll, chlorophyll a, chlorophyll b, and stomatal conductance compared to ARP1-WW plants under salinity stress (Figure 7E and Figure 10). Likewise, transgenic tobacco plants expressing *Salicornia brachiate* ribosomal protein (SbRP) ectopically displayed higher root and shoot lengths compared to wild-type plants under salinity stress. According to Udawat et al. (2017), this improvement was linked to higher amounts of carotenoids, total chlorophyll, and chlorophyll an in the transgenic plants as well as improved photosynthetic capacity.

Hence, salinization stands as a significant agricultural challenge necessitating multifaceted solutions. Utilizing reflectance indices emerges as an effective means for remotely sensing alterations in plant stress levels (Jinru & Su, 2017). Various reflectance indices exhibit sensitivity to drought (Nyongesah et al., 2014), temperature fluctuations (Osório et al., 2012), and salinization (Zinnert et al., 2012), as their values are contingent upon factors such as green biomass volume (Nguyen et al., 2020), concentrations of photosynthetic pigments (Gitelson et al., 2009). photosynthetic activity (Kior et al., 2021), LAI (Tian et al., 2017), and various other plant traits susceptible to stressors' influence. Particularly, the photochemical reflectance index (PRI) emerges as a pivotal tool in plant sensing due to its sensitivity to alterations in photosynthetic activity (Sukhova et al., 2020), even rapid changes. Notably, typical PRI proves highly responsive to soil and water salinization (Zinnert et al., 2008). Nevertheless, the directional changes in typical PRI can hinge on the type and severity of stressors (Sukhova et al., 2020), thereby constraining its

applicability in plant remote sensing. Previously, researchers introduced a series of modified reflectance indices based on diverse measuring wavelengths, demonstrating their sensitivity to excessive water deficit, light and heating. In our present study, we reveal that most of these indices (NDVI, PSRI, CRI1, WBI, NPCI, ARI1, FRI1, PRI, CCI, and greenness) also exhibit sensitivity to salinization (200 mM) (Figure 9A-J) and strong correlation with the maximal quantum yield of photosystem II (Table 4). A notable disparity in leaf spectral indices between wildtype and ARP lines was evident post salt-induced stress (Figure 9A-J). However, salinity reduced these indices in WT-NaCl and transgenic ARP1-NaCl and ARP2-NaCl, respectively, compared to their values in WT-WW and ARP1-WW and ARP2-WW plants. Furthermore, NDVI, a well-established reflectance index linked to biomass (Nguyen et al., 2020; Tian et al., 2017), LAI (Tian et al., 2017), chl content (Pastor-Guzman et al., 2015), and other slowly evolving plant parameters, underscores the influence of chlorophyll concentration on modified PRI. This influence may stem from alterations in the carotenoid-to-chlorophyll concentration ratio, which impacts typical PRI (Wong & Gamon, 2015).

According to this study, WT-NaCl plants showed unfavourable alterations in the PSII centre's structural stability, which resulted in a drop in the PSII maximal quantum yield (Fv/Fm) and performance index (PIABS) as compared to ARP1-NaCl plants. According to previous reports, plant species generally exhibit an ideal Fv/Fm ratio between 0.79 and 0.83, with lower values corresponding to stress situations (Akilan et al., 2019; Akhter et al., 2021). Therefore, a potential marker for identifying genotypes that are salt-tolerant is the kinetics of chlorophyll-a fluorescence (Kappachery et al., 2024). An in-depth analysis of OJIP-test parameters revealed that under salinity stress, WT-NaCl plants experienced more significant photoinhibition of PSII compared to ARP1-NaCl plants, disrupting electron transfer within PSII. Salt stress was less likely to affect transgenic Arabidopsis plants expressing potato StD200 than wild-type plants, which displayed lower PSII center stability and a significant decrease in the Fv/Fm ratio (Akilan et al., 2019). According to recent research, the transfer of electrons from Q_A to the electron transport chain is inhibited by salinity, which causes the plastoquinone pool (PQH_2) to decrease and light dissipation (DIo/RC) to rise more sharply (Hussain et al., 2022). A higher load was required on the reaction centers that were still active in our investigation because the salt treatment

deactivated some of the reaction centers. The energy dissipation efficiency of the remaining active reaction centers increased as a result, as seen by rising ABS/RC, TRo/RC, and DIo/RC ratios and falling ETo/RC values (Table 4). These results are in line with the effects of salinity that have been noted in *Hordeum vulgare* (Akhter et al., 2021), *Triticum aestivum* (Hussain et al., 2022), and *Raphanus sativus* (Bukhat et al., 2020). Elevated DIo/RC levels have also been associated with membrane damage, accumulation of malondialdehyde (MDA), and production of reactive oxygen species (ROS) under stressful conditions (N. Hussain, Sohail, Shakeel, Javed, Bano, Gul, Zafar, Hassan, et al., 2022). In summary, the previously described findings provide credence to the theory that ectopic expression of the ARP gene is related with improvements in PSII activity and photosynthetic pigment levels in transgenic ARP-NaCl plants, both of which are essential for photosynthesis, plant development, and survival in salty environments.

In our study, transgenic ARP1-NaCl plants exhibited a higher proline content compared to ARP1-WW plants under salinity stress, indicating maintenance of cytosolic osmotic potential in ARP1-NaCl plants (Figure 14B). Our findings are corroborated by the finding that transgenic Arabidopsis plants expressing *Withania somnifera* sterol *glycosyltransferases 3.1 (WsSGLT3.1)* displayed greater salt tolerance and increased proline content relative to wild-type plants (Mishra et al., 2021). Plants that accumulate amino acids, such as proline, are better able to withstand oxidative stress, stabilize proteins under stress, and maintain cellular turgor pressure (Kishor et al., 2013). Additionally, ARP-NaCl plants exhibited higher MDA levels and lower electrolyte leakage percentages compared to Col-NaCl plants, suggesting reduced oxidative damage in ARP-NaCl plants (Figure 14A and 8F). Similarly, Arabidopsis plants genetically modified to express *Tamarix hispida* salt overly sensitive 3 (*ThSOS3*) exhibited reduced levels of MDA and lower electrolyte leakage when subjected to high salinity conditions in comparison to their wild-type counterparts (Liu et al., 2019). Reactive oxygen species (ROS) prompt lipid peroxidation within cellular membranes, resulting in the generation of MDA as a byproduct, serving as an indicator of oxidative stress and plant antioxidant capacity (Mittler, 2002). Our findings suggest that transgenic ARP plants uphold the integrity of cell membranes amidst salinity stress by mitigating oxidative damage induced by salinity, potentially through bolstered antioxidant mechanisms. Indeed, the expression levels of

pivotal genes involved in antioxidant defense, such as APX, CAT, and SOD genes, were higher in ARP1-NaCl plants compared to ARP1-WW plants (Figure 15). These findings suggest that ectopic expression of ARP enhances transgenic plants' antioxidant capacity and helps them maintain their redox/energetic balance in the face of salinity stress. This aligns with previous research showing that ribosomal proteins either directly act as non-enzymatic antioxidants to scavenge ROS or indirectly through inducing the expression of genes that code for antioxidant enzymes (Udawat et al., 2017). Superoxide radicals ($O_2^{-\cdot}$) are converted by SOD into hydrogen peroxide (H_2O_2), which is then neutralized into water (H_2O) by APX and CAT enzymes (Gill et al., 2010).

Chapter 5: Conclusions

The result of this study revealed that transgenic Arabidopsis *ARP* plants with ectopic expression of the ARP gene had better saline stress tolerance, as seen by their longer roots than those of control plants. These findings also revealed a clear correlation between the enhanced growth performance of transgenic ARP plants and *StARP*-induced improvements in photosynthetic pigments, PSII centre maintenance, osmotic adjustment, and cell membrane integrity under salt stress. The higher salt tolerance seen in transgenic ARP plants in comparison to control plants was partly attributed to the upregulation of stress-responsive and antioxidant enzyme-encoding genes by ectopic expression of *StARP*. The impact of *StARP's* ectopic expression in *A. thaliana* on the plant's ability to withstand salt stress is thoroughly examined in our study. Based on our findings, *StARP* may be a useful gene target in the future for genetic engineering to produce crops that can withstand saline stress.

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The performance of transgenic *Arabidopsis* plants expressing *ARP1* under salinity stress was evaluated and the effect of stress conditions on photosynthetic parameters in control and transgenic plants was studied. This study has exhibited the positive role of potato auxin-repressed protein in alleviating salinity stress tolerance in higher plants.

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