IN SILICO ANALYSIS OF THE LIPID INTERACTION OF PLANT HIGH-AFFINITY POTASSIUM TRANSPORTER1 (HKT1)

A Thesis

In partial fulfillment of the award of the degree of

Master of Technology

In

Biotechnology

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(Deemed to be University)

Date: 30th June 2024

CERTIFICATE

It is certified that the entitled "In silico analysis of the lipid interaction of plant high-affinity potassium transporter(HKT1) " which is being submitted by Priya (Roll no. 302201007) in partial fulfillment of the requirements for the award of the degree of Master of Science in Biotechnology of Thapar Institute of Engineering and Technology, Patiala is a record of candidate's work carried out by her from first January 2024 to 30th June 2024 under the esteemed supervision of Dr. Debajyoti Dutta, Assistant Professor. The matter embodied in this thesis has not been submitted for the award of any other degree.

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LIST OF ABBREVIATIONS USED

At	Arabidopsis thaliana
NHXs	Na^+/H^+ exchangers
SOS	Salt overly sensitive
FAO	Food and agriculture organization
ATP	Adenosine triphosphate
BLAST	Basic local alignment searchtool
DNA	Deoxy ribo Nucleotide Acid Hydrogen
H_2O_2	Peroxide
HKT1	High-affinity K^+ transporter
MAFFT	Multiple alignment using fast fourier transform
MSA	Multiple sequence alignment
MUSCLE	Multiple sequence comparison by log-expectation
NaCl	Sodium chloride
NCBI	National center for biotechnology information
PDB	Protein data bank
OH^-	Hydroxyl radical
PM	Plasma membrane
ROS	Reactive oxygen species
RMSD	Root mean square deviation

PIP	Phosphatidyl inositol phosphate
DPPA	Dipalmitoyl phosphatidic acid
DOPE	Dioleoyl phosphatidyl ethanolamine
DPS	Di-stearoyl phosphatidyl serine
PC	Phosphatidylcholine
PI	Phosphatidyl inositol
SM	Sphingomyelin
TM	Transmembrane

ABSTRACT

Salinization is one of the most crucial soil degradation processes. Around 20 percent of the total area of the world's cultivated lands and 33 percent of irrigated agricultural lands are affected by high salinity levels. Several experimental works have been done concerning salt's effect on plants, particularly NaCl. However, these have not clarified how such an effect is linked to natural salinity and osmotic stress. Hence, salinity stress leads to ion toxicity and osmotic stresses that result in plant oxidative stress. The genes and transporters that are initiated under salt stress include Na+/H + Exchangers (NHXs), Salt Overly Sensitive (SOS), a Plasma Membrane Protein (PMP), and High-Affinity Potassium Transporter (HKT), which are involved in ion transport. The membrane transporter high-affinity potassium transporter 1 (HKT1) is vital for plant growth and tolerance to salinity. Comprehending the mechanisms plants employ to withstand the consequences of high salinity, particularly those triggered in response to disrupted Na^+ and K^+ homeostasis at the cellular and molecular levels, is crucial. In addition to helping lower Na^+-specific toxicity in plants, HKT1-type transporters maintain Na+ and K+ balance in stress conditions. An overview of the function and importance of HKT1-type transporters and their other plant species importance, particularly under the condition of salt stress, is given in this thesis. Plants can adapt to salinity in various ways, and a comparison of HKT1 structure with homologs will reveal these strategies. To understand the function of HKT1 in the membrane, we attempted its lipid interaction with protein. As a membrane protein transport, its interaction with lipids is essential. Multiple lipids docked on the HKT1 protein attempted to find the pivotal interaction between the protein and lipid.

Keywords: salinity; High-affinity potassium transporter1(HKT1); Protein-lipid interaction; Protein- lipid docking

INTRODUCTION

Salinity stress is one of the more prevailing abiotic stresses that cause substantial crop production losses, particularly in arid and semi-arid areas (Hossain et.al., 2020). According to the Food and Agriculture Organization (FAO), postulates that about 800 million hectares of land globally are affected by salinity. This widespread issue poses a significant challenge to global food security, as salinity stress can reduce crop yields, alter soil structure, and negatively impact the environment (Hernandez, 2019). Salinity is a significant problem affecting crop production worldwide: 20% of cultivated and 33% of irrigated areas are saline and sodic (Machado & Serralheiro, 2017). Salinity stress can significantly impact crop yields by reducing plant growth and development (Sarwar et.al., 2019).

Figure 1: Soil salinity development in agriculture and coastal fields. (a) Salinity in a furrow irrigated barley field, (b) Salinity in a sprinkler irrigated grass field, (c) Salinity due to seawater intrusion in coastal land (Adapted from Shahid et.al., 2018)

Soil salinity is among some of the well-known factors that cause the restriction of food crop production. Most human caloric intake comes from major staple crops like rice, wheat, and corn, which are glycophytic and cannot finish their life cycle in soil with NaCl concentrations higher than 200 mM (Zhang et.al., 2020). High levels of sodium salt in soil (approximately 225mM), often called soil salinity, can lead to salinity stress in plants **(**Hauser & Horie, 2010). This occurs when the soluble salts, including sodium salts like sodium chloride (NaCl), are too high in the soil (*Rubio et.al., 2010).* Even though NaCl is defined as the primary component of salinity, soluble salts also play a role in this process. Some of the ions of interest are calcium, magnesium, potassium, chloride, sulfate, bicarbonate, carbonate, and nitrate ions. Salinity stress occurs when the concentration of these salts surpasses the plant's capacity to withstand them, and the effects manifest in an undesirable manner on the plant's growth, development, and overall plant health. Sodium ions, in particular, are known to disrupt plant physiology by displacing essential cations like potassium, leading to ionic imbalance and osmotic stress (Munns & Tester, 2008).

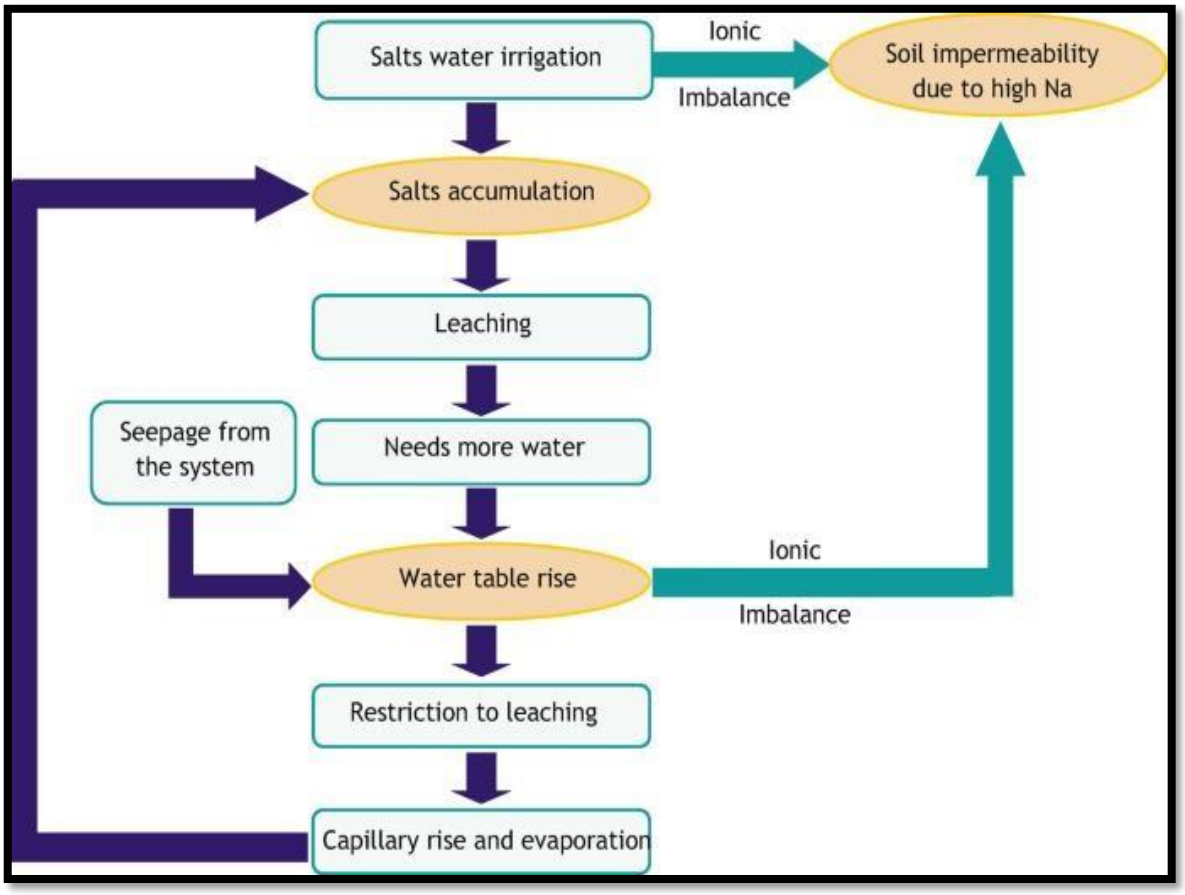

Figure 2: A hypothetical soil salinization cycle. (Adapted from Shahid et.al., 2010)

At the molecular level, sodium causes osmotic, ionic, and oxidative stress. Plants use several physiological and biochemical processes to regulate ionic balance, osmotic potential, and reactive oxygen species (ROS) homeostasis (Fu & Yang, 2023). Osmotic stress occurs when plants first encounter salt in the soil, which immediately reduces the shoot growth rate. This initial phase is primarily driven by the osmotic effect of salt, which disrupts water uptake and causes dehydration of plant cells. The second phase, ionic stress, occurs as ions such as Na^+ and Cl^- accumulate in the cytosol of cells in the shoot. This accumulation interferes with cellular processes, disrupts ion homeostasis, and can inhibit photosynthesis, protein synthesis, and other essential cellular functions. Additionally, the accumulation of ions in the cytosol can induce premature leaf senescence, further reducing plant productivity (Manuela, 2019). These stresses can lead to the malfunction of some proteins present in the cell membrane. They cause the influx of calcium incorporation, a crucial signaling molecule, into the cytoplasm. Calcium then binds to a unique protein called NADH oxidase (RBoH), triggering the production of reactive oxygen species. These ROS molecules within the cell cause oxidative damage (Mirza et.al., 2022). Plants need these proteins attached to their cell membranes to recognize and react to salt stress. Comparing the protein expression pattern under salt stress to normal conditions reveals a significant change. Most crops respond to salt stress by

either up or down-regulating the expression of the conserved set of membrane proteins. Consequently, it is possible to discover plant varieties that are salt tolerant by using differences in gene expression in plants that are stressed by salt.
Recent studies have highlighted the significance of these membrane proteins in preserving cellular integrity, osmotic balance, and ion homeostasis in salinized environments. Some transport proteins, including High-Affinity Potassium Transporter 1 (HKT1), help sodium ions exit the cell and prevent harmful build-up. Because they mediate the selective transfer of potassium and sodium ions, which is essential for reducing the harmful consequences of excessive salinity, HKT1 proteins are very important.

In addition, adaptive responses, including the production of antioxidants and Osmo protectants, are triggered by the activation or suppression of regulatory proteins engaged in signaling pathways. Plant salt resistance is emphasized by the modulation of HKT1 and related transport proteins' expression and activity in response to salt stress. As a membrane, HKT1 undergoes interaction with lipids. The detailed influence on the lipid HKT1 function and structure is not known. This work will attempt to investigate the lipid interaction with the protein HKT1.

REVIEW OF LITERATURE

Soil salinity has long been a problem for plant growth and metabolism. Thus, the issue of the salinity of soils and all its related aspects is becoming progressively more significant in our world, which is constantly evolving, both theoretically and practically. Like all other living bodies, plants undergo physiological changes when placed in unfavorable conditions of the environment. However, until very recently, the primary emphasis has been on studies relating to crop plants rather than native plants under salt stress. The biochemical and physiological properties are fundamental in developing tolerance to limiting environments. Salinity disturbs plant growth by increasing osmotic pressure and thus disturbs the normal mineral nutrition of the plants. An increased osmotic pressure reduces the plants' uptake of water, thus affecting various metabolic processes and suppressing growth. This suppressed growth has been due to the accumulation of various toxic intermediates like organic and inorganic solutes. Bernstein (1975) observed that salt solutions' effect on germination is physical and physiological. Salinity stress has been suggested to have a dual effect: a direct effect due to ion toxicity and an indirect effect due to an osmotic effect (Toole et.al., 1956; Brown, 1965; Lang, 1965).

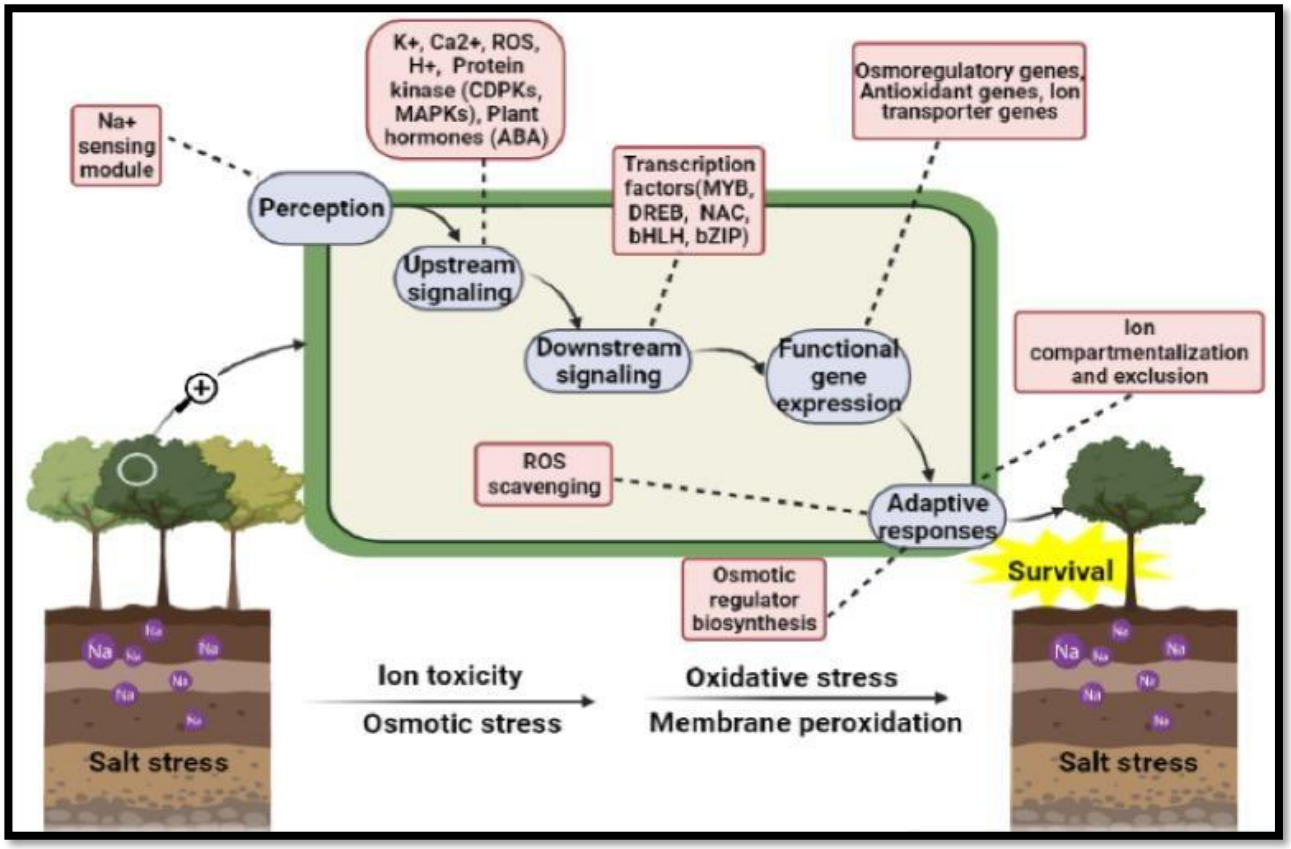

Figure 3: The process of plant salt tolerance development. (Adapted from Wang et.al., 2021)

Seed Germination of some native tropical grasses was observed to be delayed and finally suppressed by the salt stress conditions. The magnitude of reduction was found to depend upon the level of stress and the species (Sinha et.al., 1982). The suppression of nutrient absorption has also explained the

decreased growth due to salinization and the uptake of NaCl in competition with nutrient ions. As a result, salinity stress triggers cell signaling pathways (Shinozaki & Yamaguchi-Shinozaki, 2000; Knight & Knight, 2001; Zhu 2001, 2002) and cellular responses, such as accumulation of compatible solutes and up-regulation of antioxidants (Zhu et.al., 1997; Cushman & Bohnert, 2000). Solovyev (1969) found that the leading cause of NaCl-induced growth inhibition is the difficulty in the uptake of mineral nutrients due to competition with the No. At low salinity, K^+ uptake increases and decreases at higher concentrations in sugarcane (Nimbalkar & Joshi, 1980). Potassium, calcium, and magnesium in leaves of onion, celery, cucumber, and kidney have reportedly decreased, whereas in spinach, no decrease was observed under NaCl salinity. Rice accumulated more sodium and calcium indiscriminately than wheat and barley when media contained sodium chloride and calcium chloride in a ratio of 1:1 (Levitt, 1980). According to Collander (1941) and Bernstein and Hayward (1958), plants growing in saline environments generally have high sodium content. Calcium is a structural tissue component bound firmly in organic structures, and it is not an easily leachable element (Turkey, 1970). Rice is considered sensitive to salinity stress, particularly in the seedling and reproductive stages. Stunted growth, leaf rolling, white edges, drying of mature leaves, and grain sterility indicate salt stress damage in rice. Destabilization of the membrane, osmotic imbalance, and photosynthetic process disruption are the most frequent injuries (Vinod et.al., 2013; Singh et.al., 2018). Due to salt stress, rice plant water absorption is also hampered, resulting in leaf impairment (Yeo et.al., 1990; Noble & Rogers, 1992).

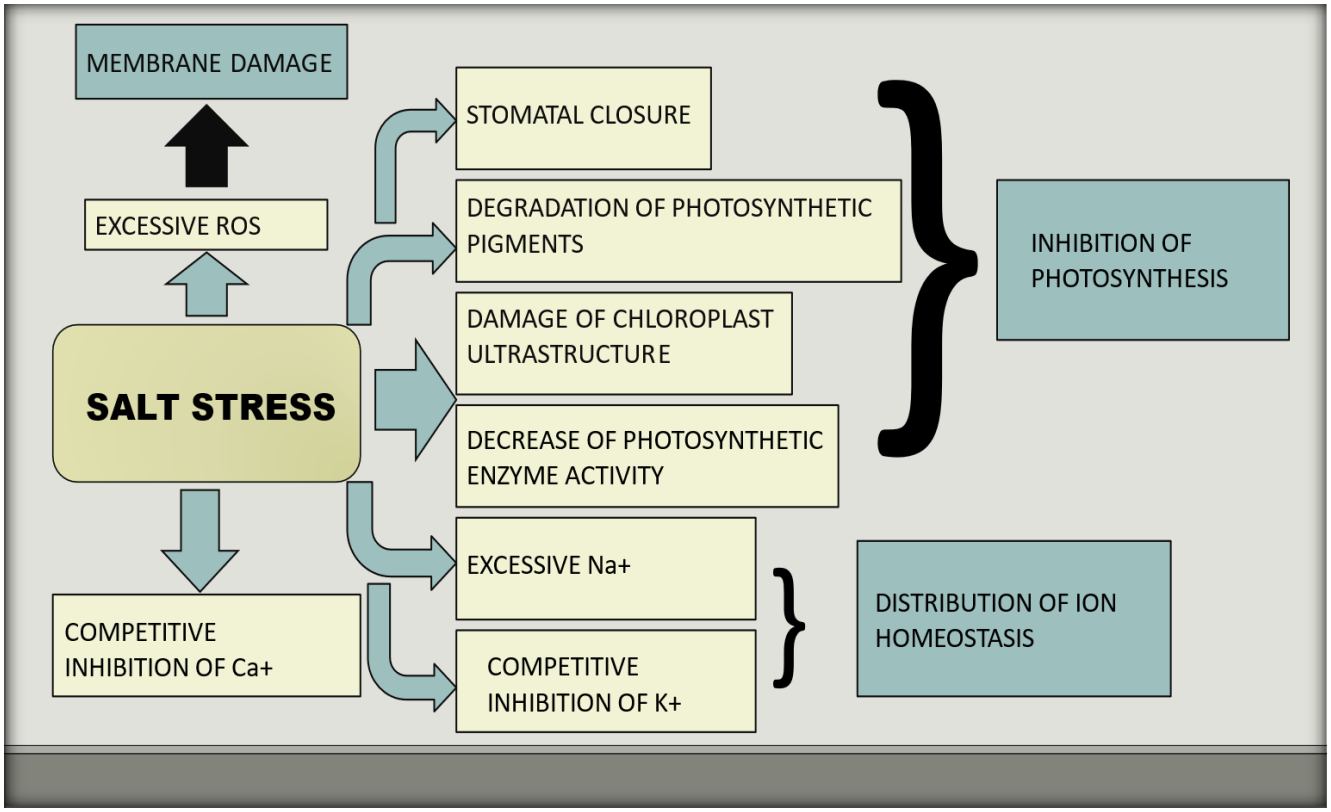

Figure 4: The mechanism of plant salt injury.

Salinity inhibits rice growth and plant development, leading to yield losses of up to 50% (Zeng & Shanon, 2000). Rice yield is reduced by 12% for every ds m-1 rise in ECe over the threshold salinity level of 3.0 ds m-1, with a 50% reduction when ECe approaches 7.2 ds m-1 (Grieve et.al., 2012). India has an 8.6 million ha salt-affected area, including 3.4 million ha of sodic soils (Sahi et.al., 2006). In the Indo-Gangetic plains, including the states of Haryana, Punjab, and Uttar Pradesh, salinity combined with water logging poses a significant danger to the agricultural sector (Babu et.al., 2017). Saline soils are reported to change plants' morphological, physiological, and biochemical responses (Amirjani, 2010; Siringam et.al., 2011). The effects of drought and salt stress on plants are very similar. Under both phenomena, the roots' absorption of water and minerals is inhibited. Among the cereals produced worldwide, 69% of the total wheat production is adversely affected by high salinity (Isayenkov, 2012). Salinity stress changes plants' physiological, morphological, and biochemical responses (Siringam et.al., 2011). It causes significant changes in the SOD (Superoxide dismutase), antioxidant enzymes, growth regulators, lipid peroxidation, total chlorophyll content, and roots and shoots fresh weight of the plant. The different physiological processes adversely affected by salt stress are mineral distribution, membrane permeability, membrane instability due to calcium displacement by sodium (Gupta et.al., 2002), and a decrease in photosynthetic efficiency (Hasegawa et al., 2000). The high-affinity K^+ transporter HKT1 facilitates plant potassium (K^+) acquisition under salinity stress conditions. Its role highlights its importance in maintaining ion homeostasis and plant growth under challenging environmental conditions (Schachtman & Schroeder, 1994). HKT1, characterized in wheat roots, transports K^+ without a high Na^+ concentration, whereas it transports Na^+ when the Na^+ concentration is high (Rubio et al., 2010). HKT transporters, found in both dicots and monocots, play a crucial role in preventing Na^+ from reaching the stem by absorbing it from the xylem sap, thereby maintaining ion homeostasis under saline conditions (Hauser & Horie, 2010). HKT1 plays a significant role in rice by regulating Na^+ homeostasis and enhancing salinity tolerance (Moreno et.al., 2016). HKT1 plays a significant role in salt tolerance in crops such as Rice (*Oryza sativa*), Barley (*Hordeum vulgare*), Wheat (*Triticum aestivum*), and *Arabidopsis thaliana* by maintaining the K^+/Na^+ ratio under salt stress conditions. According to (Garriga et.al.,2016), HKT1-mediated regulation helps plants cope with salinity by controlling sodium uptake and distribution. (Wang et.al.,2018) further highlight the pivotal role of HKT1 in balancing potassium and sodium levels to mitigate salt-induced damage. (Angessa et.al.,2020) corroborate these findings, emphasizing the importance of HKT1 in enhancing plant resilience to saline environments through effective ion homeostasis management. Specific changes in the HKT1 transporter contribute to the development of salt-tolerant rice varieties, with (Sinha et.al.,2019) emphasizing the crucial role of lipid interactions in modulating plant HKT1 function. Their research underscores how lipid-protein interactions influence HKT1 activity, shedding

light on potential mechanisms for enhancing salt tolerance in crops through genetic modifications targeting lipid interactions.

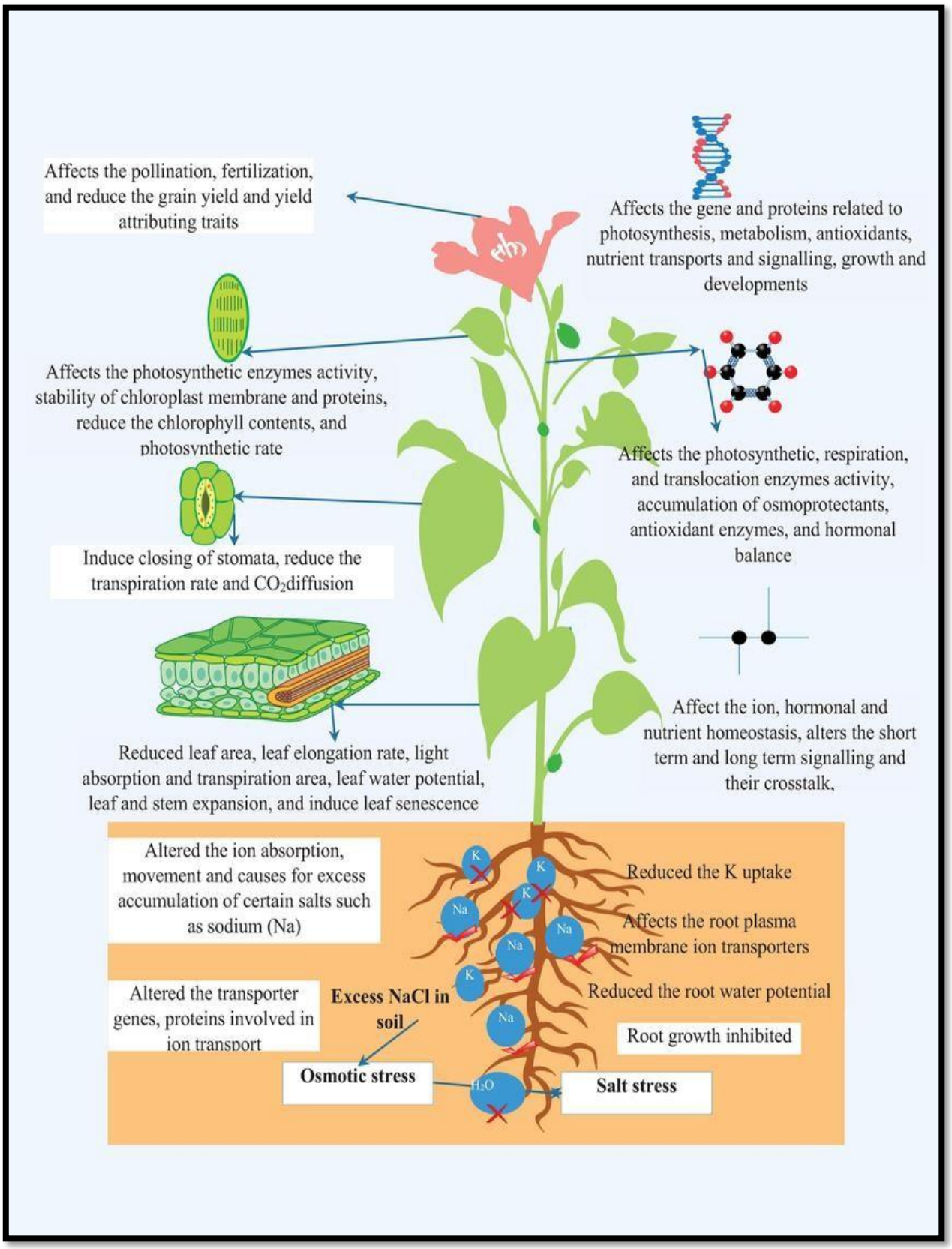

Figure 5: The effect of salinity stress on root growth, ionic homeostasis, and physiological, biochemical, and molecular processes. (Adapted from Mishra et.al., 2021)

Table 1: Different HKT genes studied in plants regarding salinity tolerance

Sl. no	Protein name	Organism name	Remark / Comments	Reference
1.	*At*HKT1; 1	*Arabidopsis Thaliana*	*At*HKT1; 1 transporter was identified as responsible for salt tolerance in Arabidopsis by modulating Na^+ accumulation and reactive oxygen species (ROS) homeostasis. Recent studies have recognized its vital role in Na^+ recirculation from xylem vessels to root xylem parenchyma cells and alleviating oxidative stress under salinity conditions. *At*HKT1;1 functions to decrease the amount of Na^+ in shoots and minimize ROS-induced cellular damage, thus overall increasing the plant's ability to cope with salt stress. These results highlight *At*HKT1;1 as a candidate gene for the genetic engineering of salt-tolerant crops needed to secure the food supply in salinized agriculture.	Ali et al. (2021)
2.	*Ta*HKT2;1 *Hv*HKT2;1 *Os*HKT2;1	*Triticum aestivum, Hordeum vulgare, Oryza sativa*	*Ta*HKT2;1, *Hv*HKT2;1, and *Os*HKT2;1 have been characterized as the K^+-Na^+ symporters genes in epidermal cells of absorption Na^+, which belongs to the HKT transporter gene family. These porters are required for the Na^+ entrance, pivotal to homeostasis in saline conditions. HKT transporters' contribution to the salt tolerance mechanisms is significant in regulating Na^+ flux, a crucial prerequisite for several cereal crops. Characterizing these transporters and unravelling their regulation, with detailed knowledge of the identity of each transporter in different species, is essential for designing crop plants that are salinization resistant. HKT in wheat, barley,	Horie, T., Hauser, F., & Schroeder, J. I. (2009)

			and rice, which are Na^+ uptake transporters involved in salt tolerance.	
3.	Skc1(*Os*HKT1;5)	*Oryza Sativa*	HKT1;5 (*Os*Skc1) transporter protein of the rice governs the K^+ uptake and salt tolerance of the Arabidopsis plant. This transporter has about 40-60% identity across plant species, and its functionality is crucial to enable the plant to adapt to salinity stress conditions. Particularly in rice, *Os*HKT1;5 has been identified as the representative for the plant's control of sodium (Na^+) distribution following salt stress to curtain the destructive impacts on growth and yield. Research has also revealed that the action of *Os*HKT1;5 includes the Na^+ transport from xylem sap in roots to the xylem parenchyma cells to minimize sodium accumulation in the shoot and the leaves. This process helps sustain the shoot K^+/Na^+ selectivity, vital for cellular processes and general plant well-being under saline stress.	Ren et al. (2005)
4.	HKT1;5 (Nax1- durum wheat) and (Kna1- bread wheat)	*Triticum Aestivum*	Triticum aestivum has salinity tolerance due to the HKT1; 5 transporter activity and some specific variations are much valued, such as Nax1 in durum wheat and Kna1 in bread wheat. These transporters are of paramount importance in maintaining the balance of sodium (Na^+) and potassium (K^+) ions that are important in the performance of plants during salinity stress. HKT1;5 (Nax1) and HKT1;5 (Kna1) help retrieve Na+ from the xylem, preventing its accumulation in the shoot and establishing a better K^+/Na^+ ratio. This mechanism is functional for the plant's cellular process and physiological health	Byrt et al. (2007), James et al. (2006)

			under salt stress. It has been found in various experiments that the mentioned transporters are highly effective in increasing the salt tolerance of wheat varieties. Therefore, they are important markers for breeding new salt-tolerant varieties of wheat.	
5.	*Os*HKT1;1(*Os*HKT1)	*Oryza Sativa*	It has been revealed that *Os*HKT1;1 transporter in the rice plant, plays an important role in the plant's coping with the salinity stress. It is targeted chiefly in Na^+ transport and has been reported to have a most important duty in Na^+ and K^+ balance in any plant. This transporter aids in the uptake of Na^+ from the xylem and reduces the Na^+ delivered to the shoots, hence minimizing the chances of sodium toxicity in the tissues of the leaves. Therefore, the plant can keep higher K^+/Na^+ ratios in the shoots, an important necessity for the cells and stress-free plant growth under a saline environment. There is scientific information that the level of expression of *Os*HKT1;1 enhances salinity tolerance in rice, and thus, the gene is a target for programs seeking to develop salt-tolerant rice varieties.	Ren et al. (2005)
6.	*Os*HKT1;5 (*Os*HKT8)	*Oryza Sativa*	There are eight highly similar HKT transporters in rice, among which *Os*HKT1;5 or *Os*HKT8 is an important Na^+ transporter that fully contributes and interactively helps the plants survive in saline stress conditions. It mainly eliminates Na^+ from the xylem, decreasing the amount of Na^+ that gets to the shoots. It assists in sustaining a higher ratio of potassium (K^+) to sodium (Na^+) in the shoots, which is desirable for normal metabolic processes and healthier plant growth. The activity of *Os*HKT1;5 plays a role in the regulation of Na^+ transport to the shoot parts, which in turn increases the salinity tolerance of the plant. A	Kobayashi et.al, 2017

			study conducted on *Os*HKT1;5 has also revealed that it can produce salt-sensitive rice varieties.	
7.	*Os*HKT1;4	*Oryza Sativa*	*Os*HKT1;4 is a sodium transporter protein in rice and is highly important to the plant's adjustment to control sodium and to endure salt stress *Os*HKT1;4 rice. This transporter is mainly located in the roots and aids in the selective uptake and transport of sodium ions; therefore, it does not allow the build-up of sodium in the plant tissues. Thus, through regulating the specific Na^+/K^+ ratio, the functioning of *Os*HKT1;4 allows the physiological processes and cell functions to be the same regardless of considering high salt concentrations in the soil. Studies done on the *Os*HKT1;4 show that it plays a crucial role in the transporters' network, providing salinity tolerance in rice.	Baumann et al.; 2012

OBJECTIVE

The present study is on plants' High-affinity K^+ Transporter 1 (HKT 1) protein, which is important for plant salt tolerance. The objectives of the study can be summarized into

- Investigating lipid interaction with plant HKT1.
- Docking of lipids on the HKT1 structure.
- Analysis of the lipid docked structure.

MATERIALS AND METHODS

4.1. *In silico* analysis

4.1.1. Tools and Software Used

- Protein Data Bank (PDB): This is used to retrieve the structures of HKT1 and its homologs.
- HHPred: For homology detection.
- PyMOL: For structural visualization, analysis, and superimposition.
- MAFFT: For sequence alignment.
- PubChem: For selecting and downloading phospholipid structures.
- OpenBabel: For converting 2D SDF structures to 3D PDB format.
- Docking Software: AutoDock for docking studies.

4.1.2. Obtaining the HKT1 protein structure responsible for plant salinity tolerance from the literature survey

A literature survey was done to find the reported HKT1 proteins responsible for the salt tolerance of different plant species. The HKT1 protein from the model plant Arabidopsis thaliana was also used in RCSB PDB to identify the 3d structure (PDB id - 8w9n). The FASTA format amino acid sequences of the HKT proteins of the model plant *Arabidopsis thaliana* were retrieved from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/) and Uniprot (https://www.uniprot.org/) database. The FASTA format sequences were downloaded. The HKT1 sequence of the plant was selected and then put in the HHpred database, and the homolog structure where lipids existed was chosen.

4.1.3. Homology Detection and Sequence Analysis

To determine the homology structure with lipid bonds available on the PDB database of protein (https://www.rcsb.org/) using the HHpred tool provides a wide range of PDB by providing a single query sequence. Homologous structures with high hit lists were identified based on percentage query coverage and E value. The best homology structure found is the one with Id 5mrw with the bound phospholipid incorporated. This putative structure provided a template for comprehending the attachment of HKT1 protein with lipid-bound membranes, so it has been valuable in understanding the protein and its regulation.

The structural data from the PDB and the homology model provided a solid foundation using HHpred, allowing further structural analysis, interaction analysis, and molecular docking.

4.1.4. Structural Superimpositions and Lipid Interaction Analysis using PyMOL

The structures of the 8W9N and 5MRW were superimposed in PyMOL software **(https://pymol.org/)** to compare their conformations and to check lipid interactions. The interactions between HKT1 (8W9N) and the phospholipid in 5MRW were analyzed. Interactions between the phospholipid and the specific amino acid were also determined: Hydrophobic and Hydrogen bond interactions. This study also identified vital interaction residues that were shown to be the same in both structures, elaborating on the role of these residues in lipid binding. The corresponding amino acid residues involved in these interactions were noted. I chain is mainly required for lipid bonds.

4.1.5. Prediction of Multiple Sequence Alignment

The sequences of HKT1 from the 8W9N structure and the homologous 5MRW were aligned using the MAFFT alignment tool (MAFFT server (https://www.ebi.ac.uk/Tools/msa/mafft/)). They revealed several conserved regions, particularly in the transmembrane domains and regions involved in phospholipid interactions. Most conserved amino acids were close to the protein's potassium ion and phospholipid-binding domains. The alignment also pointed out certain regions of variability, which could form the basis for further functional dissections of the proteins to elicit their functional contributions to the protein's activity and regulation.

4.1.6. Docking of different phospholipids with HKT1

Various phospholipids' structural coordinates were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/**)**, and their structures were downloaded in SDF format. Open Babel (https://openbabel.github.io/index.html/)was used to convert the 2D SDF structure into a 3D PDB format, making it suitable for docking studies. Autodock Vina (https://vina.scripps.edu/) and Command Prompt were used to predict the behavior of these phospholipids associated with HKT1. The docking results indicated that phospholipids interact with certain regions on HKT1, made up of residues highlighted in the interaction study.

4.1.7. Post-Docking Analysis

The docked complexes were visualized in PyMOL software **(https://pymol.org/)** to analyze the interactions between HKT1 and the docked phospholipids. The specific interactions and binding affinities were studied, including hydrogen bonds, hydrophobic contacts, and the amino acid residues involved. The interaction patterns were studied to determine how phospholipids could

regulate the structure and functioning of HKT1. The binding affinity of the docked phospholipids was calculated, providing insights into the strength and specificity of the interactions.

RESULTS

5.1 Identification of HKT1 homologue structure (in PDB) of HKT1 bound to lipids –

Arabidopsis thaliana has a single HKT1 protein. The accession number is OAO98616. This sequence has been used to find other homology structures of HKT1 by using HHpred. PDB IDs are scrutinized to investigate the presence of lipid-binding motifs. Then, the literature check confirmed that this HKT1 is essential for crop salinity tolerance. This resulted in retrieving several PDB IDs, which were visually analyzed for possible lipid-binding motifs. This entailed contrasting the structural qualities of HKT1 with other previously described lipid-binding proteins. The authors validated the lipid-binding domains necessary for the protein's function to maintain ion homeostasis under saline conditions.

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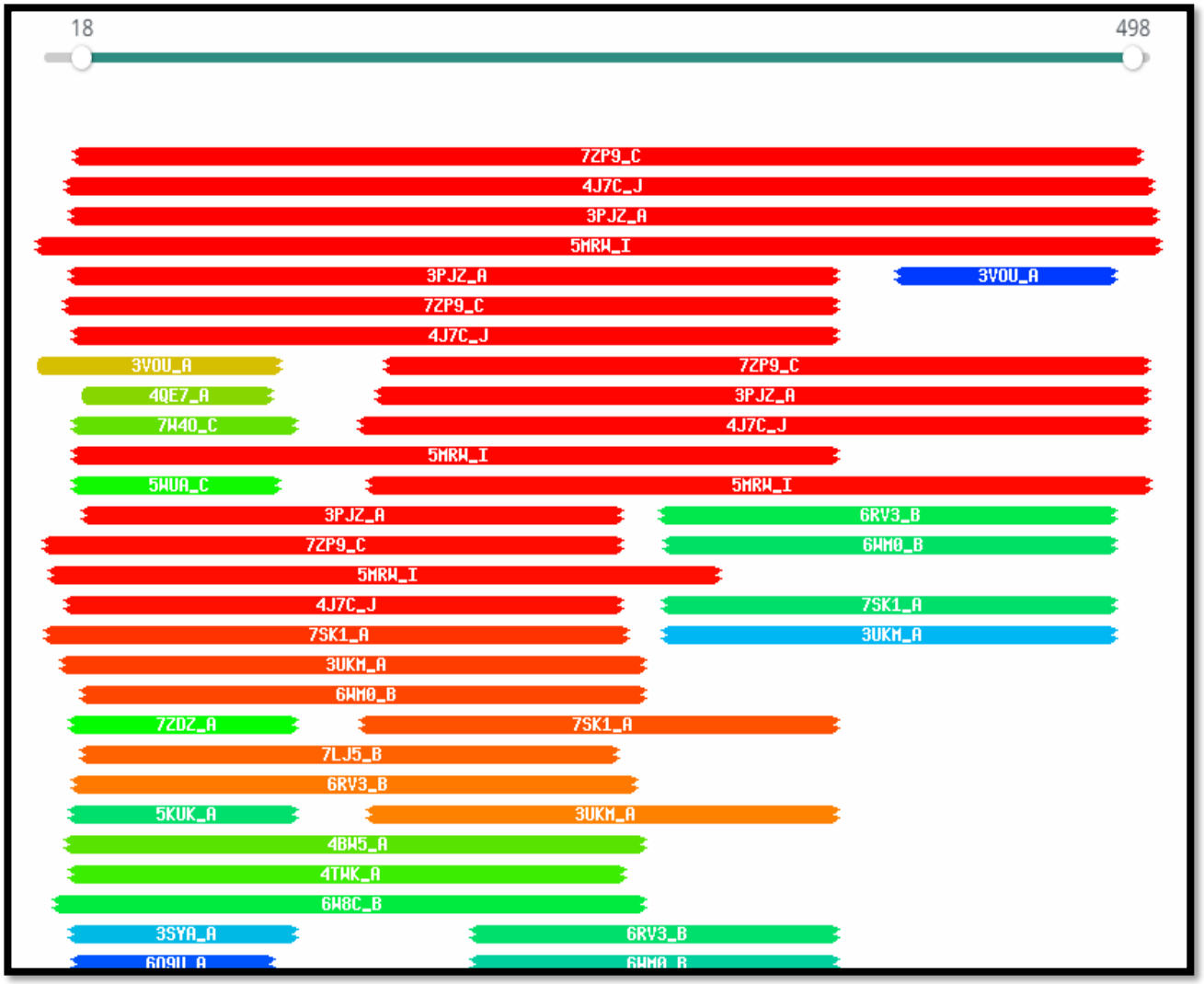
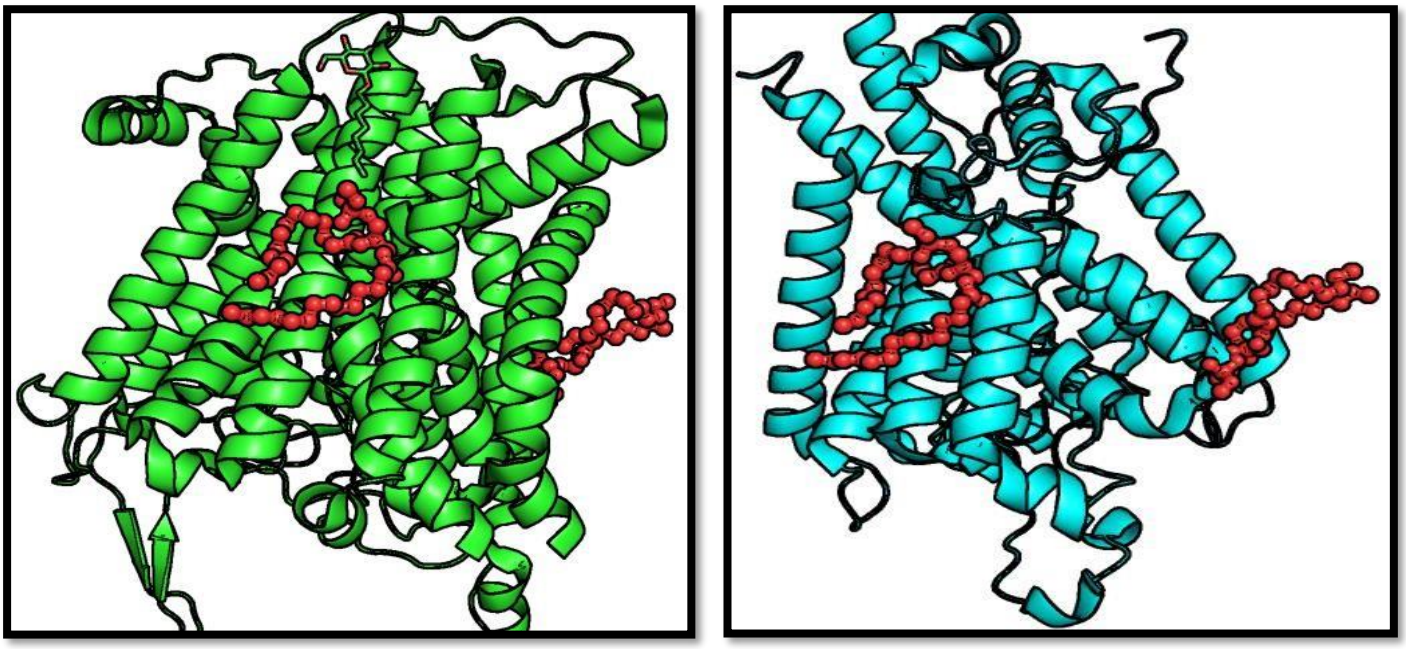


Figure 6: HHpred results showing the predicted structural homologs of HKT1 from *Arabidopsis thaliana.* Identified PDB IDs are analyzed to determine the presence of a lipid-binding domain.

5.2 Comparison of the lipid binding sites with HKT1 structure –

The features of lipid binding sites were compared between the homologous structure of PDB id 5MRW and the HKT1 structure of PDB id 8W9N. Specific conserved lipid-binding motifs were compared with HKT1 protein, emphasizing the transmembrane region associated with membrane lipids. Comparing and adjusting the orientation of the lipid bilayer by using PyMOL, several important residues and segments within the transmembrane regions were identified as critical for lipid interactions. The present results offer considerable information on these residues' structural and functional roles in HKT1(Table 2).



(A) **(B)**

Figure 7: Comparison of the lipid interactions of HKT1 homologous protein KdpFABC (PDB ID 5MRW) (A) chain I and the *Arabidopsis thaliana* HKT1 (PDB ID 8W9N) (B).

The figures above highlight the alignment of lipid-binding sites between the two chains, with red regions indicating the lipid binding sites in the green-colored Chain I of homologous protein PDB ID 5MRW (figure A) and the cyan color highlights chain A of HKT1 Structure from Arabidopsis thaliana of PDB ID 8W9N (figure B).

After each refinement cycle, the initial RMSD value was 6.85 Å, gradually converging to an improved alignment with a final RMSD of 4.068 Å.

Important residue for the lipid binding:

1. **Met360 - Ile350:**
    - **Hydrophobic Interactions:** Therefore, the fact that methionine and isoleucine are hydrophobic amino acids will largely explain how the protein can contact lipid molecules.
    - **Structural Stability:** These residues help anchor the HKT1 protein within the lipid bilayer to position it properly for optimal function.
2. **Val361- Leu353:**
    - **Hydrophobic Interactions:** The hydrophobic amino acid residues to the core include Valine and Leucine, which also contribute to the structural stability of the transmembrane region.
    - **Enhanced Lipid Interactions:** These residues are in charge of good van der Waals interactions with lipid molecules, increasing their binding and stability
3. **Trp364 – Leu356**
    - **Aromatic and Hydrophobic Interactions:** Tryptophan's side chain is aromatic and, therefore, can hydrogen bond with the lipid head groups through π-π stacking and hydrophobicity of interactions.
    - **Stabilizing Role:** Consequently, it ensures that the presence of tryptophan and leucine in the region enhances the anchoring of HKT1 to the membrane, which is critical for function.

5.3 Multiple sequence alignment and motif selection –

The sequence of HKT1 was aligned with its homolog to find the regions and the motifs of lipid interactions by using multiple sequence alignment with MAFFT (in figure 8). This alignment underlines many residues that are conserved in the sequences, thus suggesting they are to be involved in lipid interactions. Also, the same critical regions mentioned in the PyMOL analysis, Met360 - Leu352, Val361 - Phe 353, and Trp 364 - Met 356, are also underlined in the multiple sequence alignment, and details of similar amino acids can be found (in Table 3).

Important residue for the lipid binding:

1. **Met360 - Leu352:**
    - **Hydrophobic Interactions:** Methionine and leucine aromaticity makes it possible for the protein to have a strong association with lipid molecules, enhancing the stability of the protein at the cellular membrane.
    - **Structural Stability:** These residues are, in fact, central to how HKT1 is embedded within

the lipid bilayer and its orientation/operation.

2. **Val361 - Phe353:**
   - **Hydrophobic Interactions:** Valine and phenylalanine lay at the protein's hydrophobic core and help hold the transmembrane region jointly.
   - **Enhanced Lipid Interactions:** These residues contribute to van der Waals contacts with lipid molecules and improve binding affinity and stability.
3. **Trp364 - Met356:**
   - **Aromatic and Hydrophobic Interactions:** Tryptophan has a strong aromatic side chain that forms π-π interaction with the lipid head groups and hydrophobic interactions.
   - **Stabilizing Role:** Methionine helps properly link the protein to the membrane due to the interaction between tryptophan and methionine, which is essential for proper protein functioning.

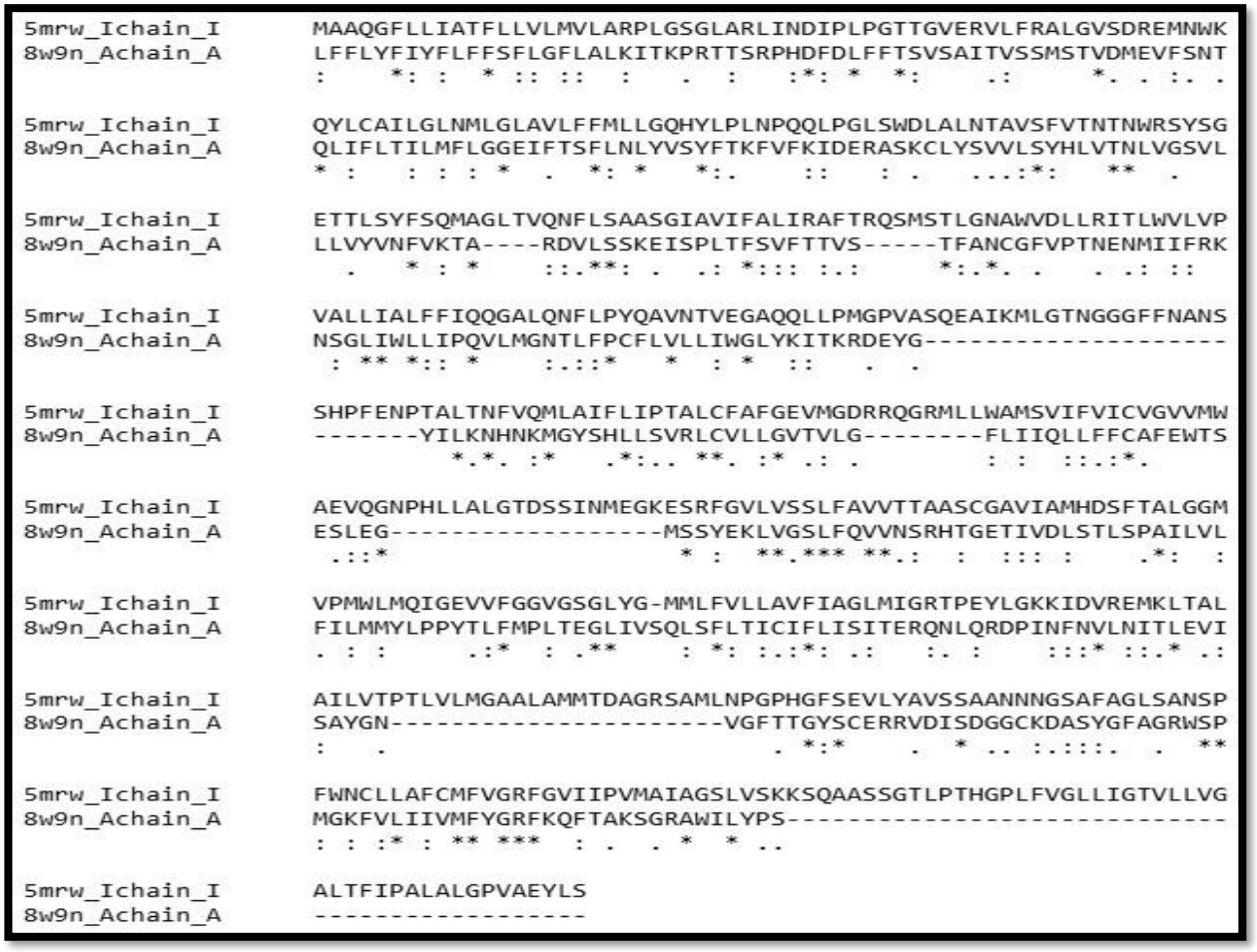


Figure 8: Multiple Sequence Alignment of HKT1 and Homologs Reveals Conserved Regions and Lipid Interaction Motifs

Structural and sequence comparison of *Arabidopsis thaliana* is done with the 5MRW template as it has a lipid-bound structure present in the PDB. Table 2 depicts the critical residue from the

comparison, and red color amino acids indicate the common amino acids responsible for lipid interaction.

Table 2: Comparison of Important Residues Involved in Lipid Binding in HKT1 from structural alignment in PyMOL and sequence alignment from MAFFT

SI No.	**Structural Alignment**		**Sequence Alignment**	
	KdpFABC residues interacting with phospholipid	Equivalent HKT1 residues interacting with the phospholipid	KdpFABC residues interacting with phospholipid	Equivalent HKT1 residues interacting with the phospholipid
`1.	Ileu290	Gly300	Ileu290	NA
2.	Ileu293	Ileu299	Ileu293	Ileu355
3.	Cys294	Phe296	Cys294	Leu233
4.	Val297	Phe303	Val297	Val236
5.	Met360	Ileu350	Met360	Leu352
6.	Val361	Leu353	Val361	Phe353
7.	Trp364	Leu356	Trp364	Met356
8.	Leu536	NA	Leu536	NA
9.	Gly533	NA	Gly533	NA
10.	Leu537	NA	Leu537	NA
11.	Ala540	NA	Ala540	NA
12.	Ileu544	NA	Ileu544	NA
13.	Leu547	Ileu355	Leu547	Trp422
14.	Val552	NA	Val552	NA

5.4 Molecular docking of lipid on HKT

Molecular docking studies were conducted to predict the interaction of selected phospholipids with the HKT1 protein. Some phospholipids such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol-4-phosphate were selected from the PubChem and were then docked onto the HKT1 structure from the Protein Data Bank using AutoDock. Hence, it is implied that analysis of the docking results in terms of binding energy and the respective bound positions was quite informative in understanding the lipid-protein interactions. The result also showed that the interaction energies of all the phospholipids were different, and the interaction pattern with phosphatidylinositol-4-phosphate had the highest binding energy (-8. 8 kcal/mol) at the dimer axis, implying its importance to HKT1 stability. PE (Phosphatidyl ethanolamine) and PC(Phosphatidylcholine), with a binding energy of -6. 8 kcal/mol and -7.5 kcal/mol. These results indicate that the dimer axis is the key lipid-binding region required for the structural integrity and function of HKT1 and, thereby, is helpful for potassium transport in plants and various salinity stress responses.

Table 3: Docking Results of Various Phospholipids with AtHKT1 (PDB ID: 8W9N)

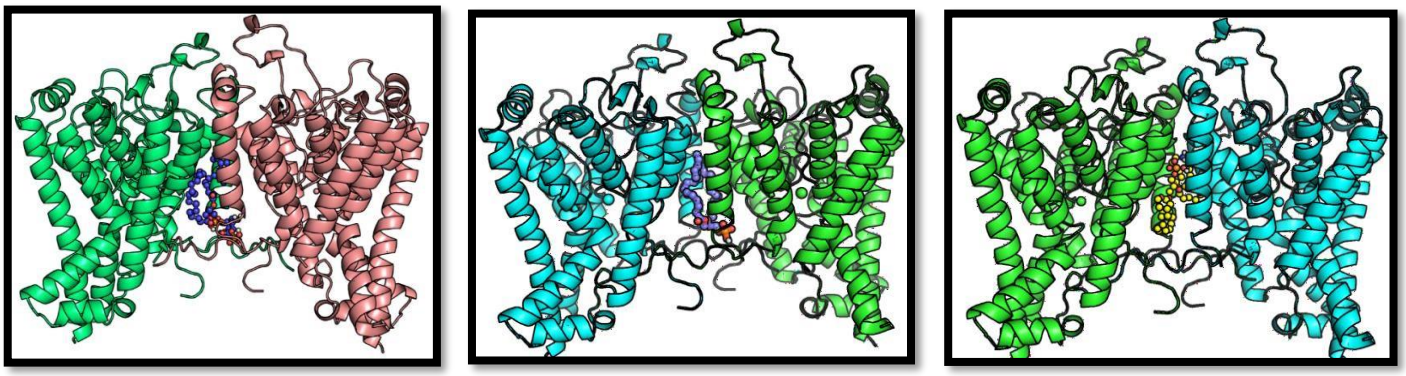
Protein Name	**Lipid Name**	**Position in the Protein Structure**	**Best Binding Energy**	**Key Interactions**
AtHKT1 PDB id – 8W9N	PIP [4'] {(17:0/20:4(5Z,8Z,11Z,14Z)} Phosphatidylinositol-4-phosphate	Dimer Axis	-8.8 kcal/mol	Hydrophobic solid and hydrogen bond interactions at the dimer axis, indicating significant stabilization of the HKT1 structure.
	PA (16:0/16:0) Dipalmitoyl phosphatidic Acid	Dimer Axis	-6.8 kcal/mol	Moderate binding energy with interactions that contribute to the structural integrity of HKT1, showing significant stabilization effects.
	PE (18:1(9Z)/18:1(9Z)) Dioleoyl phosphatidyl ethanolamine	Dimer Axis	-6.8 kcal/mol	Hydrophobic interactions enhance the stability of

				HKT1 within the lipid bilayer.
	PS (18:0/18:0) Di-stearoyl phosphatidyl Serine	Dimer Axis	-7.8 kcal/mol	Contributes to the stabilization of HKT1 through hydrophobic and hydrogen bond interactions.
	PC (16:0/18:2(9Z,12Z)) Phosphatidylcholine	Dimer Axis	-7.5 kcal/mol	Strong binding at the dimer axis is crucial for maintaining the functional conformation of HKT1.
	PI (3,5) bisphosphate (16:0/16:0) Phosphatidylinositol (3,5) bisphosphate	Dimer Axis	-8.4 kcal/mol	Similar to phosphatidylinositol-4-phosphate, showing significant stabilization effects.
	PI (4,5) diphosphate (16:0/16:0) Phosphatidylinositol 4,5-diphosphate	Dimer Axis	-8.2 kcal/mol	Effective binding at the dimer axis with strong stabilization effects.
	PIP (5') phosphate {(17:0/20:4(5Z,8Z,11Z,14Z)} Phosphatidylinositol-5-phosphate	Dimer Axis	-8.4 kcal/mol	High binding affinity and stability. .
	PI (3,4,5) {(16:0/16:0)} Phosphatidylinositol-3,4,5-triphosphate	Dimer Axis	-7.0 kcal/mol	Moderate binding energy contributes to HKT1 stability.
	SM (24:1;2O/17:0) sphingomyelin	Dimer Axis	-7.8 kcal/mol	Significant hydrophobic interactions aiding in HKT1 stabilization.

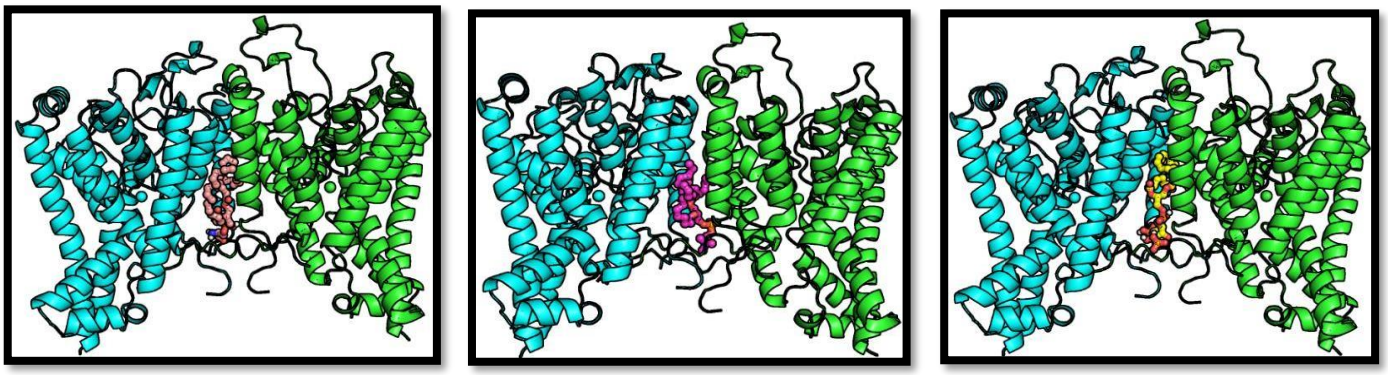
5.5 Analysis and comparison of the lipid-docked structures of HKT1:

To determine the particularities of all the distinct lipid interactions and affinities, the various structures of HKT1 with different lipids docked were considered. The binding sites and interaction patterns were compared to define the features that are the same and those that are different for each lipid. The docking application generated output files in PDBQT format, and molecular visualization was done in PyMOL to identify the lipid binding sites and their respective positions.

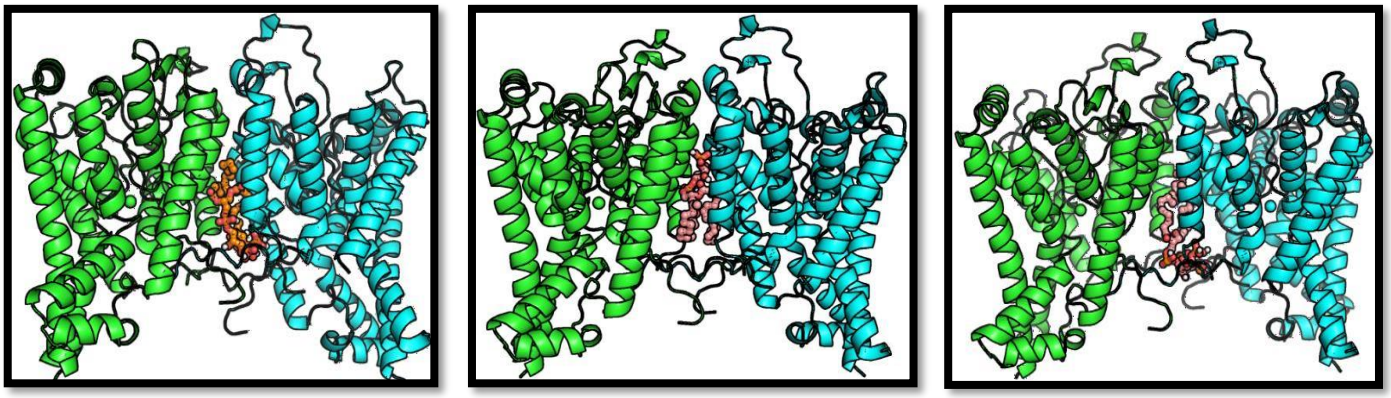
Different lipids are used to check which lipid is firmly attached to the protein PDB ID- 8W9N from *Arabidopsis Thaliana*. The molecular docking study of the high-affinity potassium transporter 1 (HKT1) from *Arabidopsis thaliana* (PDB ID 8W9N) presents different lipids involved in protein stability from Figures 9A–9J shown below.



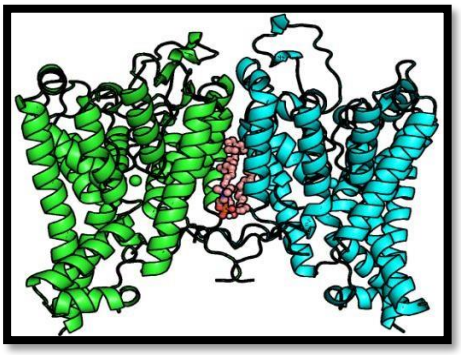
(A) (B) (C)



(D) (E) (F)



(G) **(H)** **(I)**



(J)

Figure 9: Lipid molecules docked on the HKT1 structure. Lipids phosphatidyl inositol-4-phosphate**(**A), dipalmitoyl phosphatidic acid (B), dioleoyl phosphatidyl ethanolamine(C), di-stearoyl phosphatidyl Serine(D), phosphatidylcholine(E), phosphatidyl (3,5) inositol bisphosphate(F), phosphatidylinositol 4,5-diphosphate(G), phosphatidyl inositol-5-phosphate(H), phosphatidyl inositol-3,4,5-triphosphate(I), sphingomyelin(J) manifests a consensus docking on the protein at the dimer interface

DISCUSSION

High-affinity plants' K^+ transporters (HKTs) are essential transmembrane transporters in maintaining the equilibrium of Na^+ and K^+ ions in plants, impacting plant cytosol under K^+-deprived conditions and increasing salinity tolerance.

This study aimed to understand the mode of interactions between the lipids and the HKT1 transporter from the organism *Arabidopsis thaliana*, which is essential for plant salt tolerance. The objectives were to obtain the lipid-bound structures using the computational docking method and to postulate the lipid effects on HKT1 activity and structure. Sequence homology of *A. thaliana* HKT1 with the existing PDB structures showed that only one lipid-bound homologous structure is present. Structural superimposition between these two proteins showed an RMSD value of 4.06 Å, endorsing a considerable homology. In the homologous structure KdpFABC transporter domain (PDB ID 5MRW chain I), critical residues are found when the protein is compared with the HKT1. Some critical residues in HKT1 are I350, L353, and L356, which may be crucial for interacting with the lipids. These interactions stabilize the protein and enhance its affinity for lipid binding, which is critical for its function. With the help of Pymol, the analysis ensured the position and interaction of lipids and emphasized the role of specific residues in lipid binding.

Different lipids were further computationally docked on HKT structure to understand the lipid-bound structure (PDB 8W9N). For the same purpose, lipids such as PIP (phosphatidyl inositol-4-phosphate), PC (phosphatidylcholine), PE (phosphatidyl ethanolamine), and many more are one by one docked on the protein. Molecular docking further supported these interactions to expose the interaction and binding affinity. The interaction between protein HKT1 and its ligand was ranked primarily to binding energy, divided into three groups. In the first group, phosphatidyl inositol-4-phosphate has the highest binding energy, -8.8 kcal/mol. In the second group, phosphatidylinositol (3,5) bisphosphate, phosphatidylinositol 4,5-diphosphate, and phosphatidylinositol-5-phosphate, di-stearoyl phosphatidyl Serine, and sphingomyelin have binding energy from 7.8-8.4. In group 3, phosphatidylcholine, phosphatidylcholine, phosphatidylinositol-3,4,5-triphosphate, PA (dipalmitoyl phosphatidic Acid), and PE (dioleoyl phosphatidyl ethanolamine), have the lowest binding energy from 6.8-7.0. The common feature discovered by analysing the docking is that all the lipids are bound to the dimer interphase of the protein. The dimer interphase is located between the two molecules of the HKT1. It contains a 1066cm^3 volume interdimer space occupied by lipids. Most of the lipids are found in the inner leaflet of the membrane. The lipid head group is oriented towards the interior or cytoplasmic side of the structure that is part of the inner leaflet of the membrane. In all cases, it was found that lipids occupy the space between two monomers of the protein at the dimer interphase. From the analysis, it has been found

that most of the common residues involved during the lipid interaction are Ser398, Tyr359, Leu400, Thr404, and Gly399. The dimer interphase is essential for the localization of the protein.

Based on our findings, we inferred that the idea that lipid-protein interaction is vital for membrane protein functionality is accurate, as presented in other related studies. About specific lipid-binding residues and motifs of HKT1, the study is parallel to revealing how membrane lipids participate in protein stability and activity. The lipid-binding motifs and their relations are crucial for the HKT1 structural stability and functionality so plants can survive in high saline conditions. Knowledge of these interactions gives potential sites for genetic engineering to increase crop tolerance to salinity stress. Therefore, based on this study, lipid-protein interactions are essential to the functionality of HKT1. Through molecular docking, sequence alignment, and homology detection, this study has offered a foundation to address the subsequent research directions by modifying HKT1 and its lipid interactions for protein function and stability and enhancing crop resistance to salinity. Future experimental investigations are still needed to support these in silico studies and investigate possible agricultural biotechnology applications. Considering other possible lipids that may interact with HKT1 and the observed impact on its function, further research could increase the knowledge about plant salt tolerance.

CONCLUSION

The HKT1 protein thus has an essential function in plants by controlling the movements of sodium (Na^+) and potassium (K^+) ions. It assists in regulating ion balance and coping with salt stress challenges. Thus, HKT1 regulates the uptake and distribution of Na^+, thus helping plants to withstand high salinity levels and grow in conditions associated with high salt content.

This in silico study of lipid interactions with HKT1 from *Arabidopsis thaliana* gives significant information on lipid-binding residues' structural and functional relevance. Using homology detection with HHpred, multiple sequence alignments with MAFFT, and molecular docking simulations with AutoDock vina, the lipid-binding motifs and their access to the motif were unique. They proved to be significant in the maintenance of ion balance in response to saline stress. Using HHpred, it was possible to identify PDB structures homologous to HKT1, which helped predict and validate lipid-binding sites that are further used for future purposes to check the lipid interaction of HKT1 structure from Arabidopsis thaliana by using PyMOL software. Some of these critical residues were also found, including M360-I350, V361-L353, and W364-L356, for the superimposition of the HKT1 protein within the lipid bilayer through interactions, which improve the structural stability of the protein and its lipid binding affinity. Molecular docking also pointed to the dimer axis as the lipids binding site; the first and the most preferred lipid was phosphatidylinositol-4-phosphate, with a binding energy of 8.8 kcal/mol. This was consistent with the visualization done with PyMOL, which depicted the position of the lipids and their interaction, thereby showing that specific residues are essential in lipid binding. Thus, the presented data must underline the critical role of lipid-protein interactions in the functionality of HKT1, its participation in potassium transport in the plant, and tolerance to the action of salt content. The lipid-binding motifs and interactions mentioned earlier are crucial for the structural stability and proper functioning of HKT1, allowing plants to grow and thrive in high saline conditions. From this research, there are candidates for genetic intervention that can increase the tolerance of crops to salinity stress. Overall, this thesis establishes the importance of lipid-protein interactions in the functionality of the HKT1 channel. Therefore, the first and most crucial step in achieving salinity tolerance is understanding lipid-binding residues' structural and functional features by identifying and validating them. This will facilitate subsequent studies on crop salinity tolerance by controlling the expression of HKT1 and lipid engagement. Nevertheless, these in silico findings must be validated with more experiments, and more potential applications in agricultural biotechnology must be investigated.

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