Overexpression and Purification of *Arabidopsis thaliana* HKT1;1 protein in yeast *Pichia pastoris*

A thesis submitted in partial fulfillment of the requirements for the award of the degree

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

Submitted by

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Under the guidance of

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DECLARATION

I, Rishabh hereby declare that the work presented in the thesis "Overexpression and Purification of *Arabidopsis thaliana*HKT1;1 protein in yeast *Pichia pastoris"* in the partial fulfillment of the requirements of the award of the degree of Masters of Science in Biotechnology at Thapar Institute of Engineering and Technology (TIET), is an original and genuine work completed by me between Jan 2026 and July 2026. This research was carried out under the guidance and supervision of Dr. Debajyoti Dutta, Assistant Professor in the Department of Biotechnology, TIET. The content presented in this thesis has not been previously submitted, either in its entirety or in part, to any other educational institution or university in India or abroad for the purpose of obtaining any degree.

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CERTIFICATE

This is to certify that thesis entitled **"**Overexpression and Purification of *Arabidopsis thaliana*HKT1;1 protein in yeast *Pichia pastoris*" submitted by **Mr. Rishabh (Roll no. 3024010051)** in the partial fulfillment of the requirements for the award of the degree of **Master of Science in Biotechnology, Thapar Institute of Engineering and Technology, Patiala** is a record of the student's own work carried out under my guidance and supervision. This work has not been submitted in part or in full to any other university or institute for the award of any other degree.

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ACKNOWLEDGEMENT

I am deeply grateful to the many individuals and institutionsfor supporting me throughout this thesis journey. Their encouragement, guidance, and practical assistance made this work possible.

I extend my heartfelt thanks to my thesis supervisor, **Dr. Debajyoti Dutta**, for his expert guidance, thoughtful feedback, and constant encouragement. His mentorship has been invaluable in shaping this research and my growth as a scientist.

I sincerely thank **Dr. Manoj Baranwal,** Head of the Department, for granting me permission to undertake this thesis.

A special note of gratitude goes to **Ms. Sanhita Banik**, whose unwavering support, prompt responses, and willingness to help with every query and challenge kept this work on track. Her dedication has been a cornerstone of this thesis.

I also acknowledge the skilled assistance of**Mr. Ram Newal Yadav,**laboratory superintendent, and laboratory technicians;their technical help and maintenance of the lab environment were essential for the smooth conduct of experiments.

I am thankful to my fellow lab member **Aayush Banerjee** for his camaraderie and helpful discussions. I also extend my appreciation to PhD scholars**Mr.Sambit Majumdar** and **Mrs**.**Tanya Bhatnagar** for their cooperation during this work.

Finally, I would like to thank myself for staying persistent, overcoming setbacks, and completing this thesis. I also remain grateful to my family and friends for their unwavering belief in me.

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Abstract

Salinity is one of the most important abiotic stresses on about 20% of the world's irrigated areas. It is a serious issue that compromises world food production. Salt tolerance in plants depends on the regulation of ion homeostasis by membrane transporters AtHKT1;1 of *Arabidopsis thaliana* is one such crucial transporter. It helps in the retrieval of Na^+ from the xylem sap to safeguard photosynthetic tissues. AtHKT1;1 has been physiologically validated in several organisms, but there is no report of its functionality in *Pichia pastoris*, a scalable platform that is ideal for biochemical and structural studies. The purpose of this thesis was to heterologouslyexpress, purify and detect AtHKT1;1 in *Pichia pastoris* GS115. Transformed cells with the pPICZ-A-*athkt1;1*construct was grown in BMGY medium, induced with methanol for 72 h and harvested. Two parallel membrane isolation protocols were tested: direct Sonication followed by solubilization and Zymolyase mediated spheroplast formation followedby Sonication and solubilization. His-tagged protein was prepared using Ni-NTA affinity column and eluted with 300 mM imidazole. The level of protein expression and purification was tracked by SDS-PAGE and Western blotting with anti-His and anti-eGFP antibodies. Elution fractions from both isolation techniques showed a band corresponding to the expected molecular weight on Coomassie-stained gels and Western blots, and the isolation and purification of AtHKT1;1 in *Pichia pastoris* were successful. The purified protein was kept at –80 °C for future studies. This work represents the first functional validation of AtHKT1;1 produced in *P. pastoris* and includes a reproducible pipeline for production of AtHKT1;1 that enables further analysis, including interactome mapping and structural characterization.

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List of Abbreviations

Abbreviation	Full Form
ABA	Abscisic Acid
AOX1	Alcohol Oxidase 1
APS	Ammonium Persulfate
AtHKT1;1	*Arabidopsis thaliana* High-Affinity K^+ Transporter 1;1
BCA	Bicinchoninic Acid
B-Me	β-Mercaptoethanol
BMGY	Buffered Glycerol-complex Medium
BMMY	Buffered Methanol-complex Medium
cryo-EM	cryo-Electron Microscopy
DAB	Diaminobenzidine
eGFP	enhanced Green Fluorescent Protein
EDTA	Ethylenediaminetetraacetic Acid
FAO	Food and Agriculture Organization
FT	Flow-Through
HKT	High-Affinity K^+ Transporter
HRP	Horseradish Peroxidase
IMAC	Immobilized Metal Affinity Chromatography
Mut^+	Methanol Utilization Positive
Mut^s	Methanol Utilization Slow
NaCl	Sodium Chloride
NHX	Na^+/H^+ Exchanger
Ni^{2+}-NTA	Nickel-Nitrilotriacetic Acid
$NiCl_2$	Nickel Chloride
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-Buffered Saline
PDB	Protein Data Bank
PTM	Post-Translational Modification

PVDF	Polyvinylidene Difluoride
QTL	Quantitative Trait Locus
ROS	Reactive Oxygen Species
rpm	Revolutions Per Minute
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate
SEC-MALS	Size-Exclusion Chromatography with Multi-Angle Light Scattering
SMA	Styrene-Maleic Acid Copolymer
SMA200	Styrene-Maleic Acid Copolymer (2:1 ratio)
SMA2000	Styrene-Maleic Acid Copolymer (alternative notation)
SMALP	Styrene-Maleic Acid Lipid Particle
SOS	Salt Overly Sensitive
TBST	Tris-Buffered Saline with Tween-20
TEMED	Tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
WT	Wild-Type
YPD	Yeast Peptone Dextrose

List of symbols

Symbol / Unit	Meaning
%	Percent
°C	Degree Celsius
×g	Times gravity (centrifugal force)
g	Gram
h	Hour
kDa	Kilodalton
L	Litre
M	Molar
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
mm	Millimetre
nm	Nanometre
pH	Potential of hydrogen
rpm	Revolutions per minute
s	Second
µL	Microlitre
µM	Micromolar
V	Volt
W	Watt

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CHAPTER 1

INTRODUCTION

1.1 The Global Challenge of Soil Salinity

Salinity is a major abiotic stress that impacts around 20% of the world's irrigated soils. It poses a serious threat to global food security (FAO, 2021). Soil salinity is increasing at an alarming rate due to unsustainable irrigation, sea-level rise, and climate change, and has become essential in agriculture to develop crops that are tolerant to salt. Increased resistance and sustainable productivity are vital on increasingly marginal soils.

Fig. 1: Visual from "Evaluation Matters" by C. J. Copp and N. Elgeberi, 2025, University of Nevada, Reno Extension

1.2 Ionic Toxicity and Plant Adaptation to Salinity

The main way that salt stress affects cellular ion homeostasis is through sodium (Na^+) toxicity (Munns & Tester, 2008). A rise in Na^+ causes a corresponding decrease in the cytosolic K^+, which is required for the function of the enzymes involved in protein synthesis, photosynthesis, and stomatal function (Shabala&Pottosin, 2014). Because sodium cannot replace potassium in these functions, a phenomenon known as oxidative stress, metabolic inhibition and the classic symptom of salt injury occur.Plants have a coordinated transport system responsible for the careful regulation of Na^+:

• Vacuolar sequestration isolates Na^+ from the cytosol, using proton gradients generated by the V-ATPases and V-PPases to drive transport via NHX exchangers (Bassil&Blumwald, 2014).

• Plasma membrane extrusion mediated by SOS1 Na^+/H^+ antiporters limits Na^+ entry into roots (Shi et al., 2000).
• Long-distance regulation is achieved through HKT transporters, which get the Na^+ from the xylem sap, reducing shoot Na^+ accumulation and protecting photosynthetic tissues (Platten et al., 2006).

1.3AtHKT1; 1: A Key Protein for Sodium Homeostasis

In *Arabidopsis thaliana*, the well-characterized Subfamily I HKT transporter AtHKT1;1 is a sodium-selective uniporters. It is mainly expressed in the root stele cells and xylem parenchyma and it removes sodium from the xylem and reduces its translocation to shoots in an important mechanism for salt tolerance (Sunarpi et al., 2005). Loss of function AtHKT1;1 mutants accumulate more Na^+ in shoots and results in restricted growth under salt stress, highlighting the physiological role of this solute in salinity stress. However, there is a gap that remains: thatthere are no studies of AtHKT1;1 protein production and purification using yeast *Pichia pastoris*, which can help us to extend this study towards the understanding of the protein and lipid interactome of AtHKT1;1.

1.4 The Need for Heterologous Expression

Pichia pastoris sits in a spot that makes it particularly well-suited for plant proteins, especially membrane proteins, for a few converging reasons. It is a eukaryote and uses similar general protein-folding machinery as plant cells, such as the ER folding, disulfide bond formation, and correct post-translational processing (Cereghino&Cregg, 2000). Bacterial hosts like *E. coli*, however, do not possess this capability, and often do not make functional eukaryotic membrane proteins (Grisshammer& Tate, 1995). *Pichia* is much more cost effective, faster and easier than other eukaryotic systems (insect or mammalian cells) and can produce much higher yields (Macauley-Patrick et al., 2005). It can achieve expression levels 10 - 100 fold higher than *S. cerevisiae* (Cereghino&Cregg, 2000). Structurally complex membrane proteins such as ion channels and transporters have been successfully expressed in *Pichia* (Byrne, 2015; Villalba et al., 2005).*Pichia* has a balance between the provision of both eukaryotic folding and secretory machinery together with the ability for high density, cost-effective fermentations with the strong and inducible AOX1 promoter provided by the methylotrophic yeast, *Pichia pastoris.*It is an excellent host for the expression of functional plant ion transporters, to be used as a host for expression of AtHKT1;1.But it matters not if successful, whether or not this is sufficient for function. Biochemically verifying that the recombinant protein is both present and active is crucial. As such, functional validation, signaling the connection of the protein detection to the measurable physiological phenotype of the host is commonly under-explored and a necessary component to validate the further effort to purify or structure the protein.

1.5 Research Gap and Project Aims

Although the genetic role of AtHKT1;1 salt tolerance in Arabidopsis is well established, its functional role in *Pichia pastoris* is unknown. There are no studies of AtHKT1;1 protein production and purification using yeast *Pichia pastoris*.

Objectives: The main objective of this project is to functionally validate AtHKT1;1 expressed in *Pichia* pastoris and is the following:

Objective 1: Overexpression of AtHKT1;1 in *Pichia pastoris*

Objective 2: Isolation & Purification of AtHKT1;1 over-expressed in *Pichia pastoris*

This work will offer crucial genetic validation of *Pichia*-derived AtHKT1;1, to correlate with protein-level function and lay the groundwork for later structural and mechanistic investigation into this important salinity-tolerance determinant protein.

CHAPTER 2

Literature Review

2.1 The Global Challenge of Soil Salinization

2.1.1 Extent and Impact on Agriculture

Soil salinization is one of the most severe threats to global agricultural productivity as it affects approx20%of irrigated land worldwide and results in economic losses all over the world (FAO, 2021). The problem in particular is acute in arid and semi-arid areas where high evapotranspiration rates concentrate salts in the root zone. According to the Food and Agriculture Organization of the United Nation, over 833 million hectares of land are salt affectedglobally, with Asia and Australia being the most severely impacted continents (FAO, 2021). Secondary salinization resulted from improper irrigation practices that result for about 77% of this total, highlighting the contribution to this environmental crisis. The impact on crop production is deep and has different aspects asmajor cereal crops exhibit different degrees of sensitivity such as with rice, it is particularly vulnerable due to its growth in flooded conditions that increase salt accumulation (Ismail &Horie, 2017). Wheat shows moderate tolerance and barley shows relatively high resilience. The Physical signs indicate 30-50% yield reductions in affected areas and complete crop failure occurring under severe conditions (Munns &Gilliham, 2015).

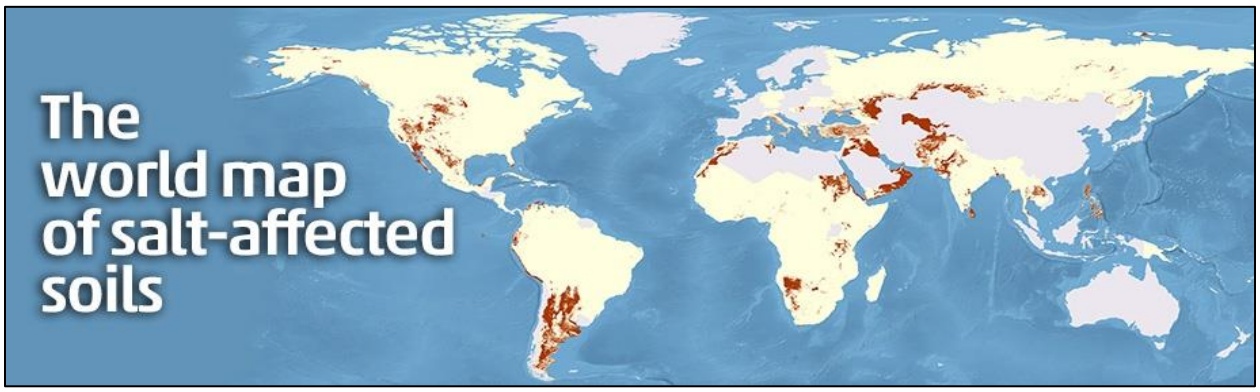

Fig. 2: Global distribution of salt-affected soils. [Source: FAO, 2021]

2.1.2 Geographic Distribution and Climate Change Interactions

Hotspots for salinity are distributed across specific geographic areas with different factors such as the Indo-Gangetic plain, which is considered South Asia's breadbasket, but it faces increasing salinization due to intensive irrigation with marginal quality water and rising water tables (Singh, 2018). In the Middle East and North Africa, where 90% of agriculture relies on irrigation, high temperatures and the limited freshwater resources add to salt accumulation in soils. Climate

change is a threat as it increases soil salinization through several factors, such as rising sea levels contributing to saltwater intrusion in coastal areas, while increased frequency of extreme weather events alters precipitation patterns and evaporation rates (IPCC, 2022). Models predict that under current emission scenarios, an additional 50-100 million hectares of land could become salt-affected by2050, disproportionately impacting developing nations with limited adaptation capacity (Hassani et al., 2020). This convergence of environmental challenges underscores the urgency of developing salt-tolerant crops as a climate adaptation strategy.

2.1.3 Socio-economic Implications

The impact on food security extends beyond just yield losses asit affects food availability, access, and stability. In salinity affected regions of South Asia, per capita food production has declined by 15–25% over recent decades, contributing to continuous malnutrition and food insecurity (FAO, 2022). The situation is particularly acute in countries with limited arable land and high population density, where every hectare lost to salinization represents a permanent reduction in food production capacity. Global food systems face increasing pressure as productive agricultural land diminishes while the population continues to grow, with projections indicating that food production must increase by 60% by 2050 to meet demand (Alexandratos&Bruinsma, 2012). Water resource management challenges lie at the heart of the salinity problem. Inappropriate irrigation practices, such as the use of saline groundwater without adequate drainage, have created secondary salinization across millions of hectares (Qadir et al., 2014). Competing demands for freshwater from urban, industrial, and environmental sectors reduce water availability for agriculture, forcing farmers to utilize increasingly marginal water sources. Groundwater depletion in major agricultural regions like the Indo-Gangetic Plain and North China Plain exacerbates salinity by concentrating salts in remaining water and drawing deeper, more saline water into the root zone (Rodell et al., 2009).

2.2 Plant Salt Tolerance: From Whole Plant Physiology to Cellular Mechanisms

2.2.1 Classification of Plant based on Salinity

Plants are divided into halophytes and glycophytes based on their capacity to deal with salt stress. Halophytes (~1% of plant species) possess specialized adaptations, that is salt glands, succulent tissues, and enhanced ion compartmentalization, which enablesurvival in >200 mM NaCl (Flowers &Colmer, 2008). In contrast, glycophytes (most crops: rice, wheat, maize) lack these structures and show damage at 50–100 mM NaCl (Munns & Tester, 2008). The fundamental distinction lies not in unique genes but in the regulation and coordination of stress responses. Halophytes maintain lower cytosolic Na^+ through more efficient exclusion and compartmentalization, coupled with enhanced antioxidant capacity and compatible solute synthesis (Flowers et al., 2010). Notably, many tolerance mechanisms exist in glycophytes but operate with reduced efficiency or improper regulation, providing a rationale for engineering

improved salt tolerance by enhancing existing pathways rather than introducing entirely novel systems (Munns et al., 2016).

Plants employ two primary strategies for managing salt stress: Tissue tolerance and Ion exclusion. Tissue tolerance involves vacuolar ion sequestration, allowing accumulated ions to be used for osmotic adjustment while protecting cytoplasmic metabolism (Flowers &Colmer, 2008). Ion exclusion minimizes Na^+ uptake at the root level and restricts shoot translocation, preventing toxicity in photosynthetic tissues; this is the dominant strategy in most glycophytes (Munns & Tester, 2008). The balance between these strategies varies among species, cultivars, and developmental stages. Developmental stage-specific sensitivity adds further complexity, seed germination and early seedling establishment are highly vulnerable due to osmotic effects and ion toxicity (Ungar, 1995), while reproductive development, particularly flowering and grain filling, suffers disproportionate yield losses even when vegetative growth appears moderately maintained (Läuchli& Grattan, 2007). Understanding these stage-specific sensitivities is essential for designing effective screening protocols and targeting genetic improvements to the most critical developmental phases.

2.2.2 Time Course of Salt Stress Responses

Plant adaptation to salinity occurs in a series of time-dependent stages controlled by various physiological and molecular processes (Munns & Tester, 2008). The immediate osmotic phase starts a few minutes after exposing of the cell to salt; this is characterized by rapid water loss and reduced cell expansion. This is the first signal that is mediated by ABA-dependent signaling that causes stomatal closure, reducing photosynthesis, and altering gene expression (Cutler et al., 2010). The inhibition of growth during this stage is mainly caused by water deficit in comparison to ion toxicity and the shoot growth is more severely affected than the root growth an adaptive response that might save water and yet retain the ability to explore the soil water table (Munns, 2002).The ionic phase is formed within days to weeks as the deposition of the Na^+ ions reaches dangerous levels in transpiring leaves (Munns & Tester, 2008). In this time ions that had entered the plant during the initial uptake of water are concentrated as the process of transpiration progresses and are not diluted. Older leaves are affected first, as the older leaves have been transpiring the longest and have accumulated the greatest amount of salt. Ion toxicity leads to the progressive weakening of photosynthetic capacity due to damage of chloroplasts and inhibition of key enzymes (Munns et al., 2006). The range of stress intensities, plant species, and environmental conditions where the transition from osmotic to ionic phase becomes obvious varies, although in most cases it becomes evident within 7–14 days under moderate salinity.Adaptation and recovery processes over the long term enable certain plants to recreate homeostasis following short-term reactions to stress (Zhu, 2002). This adaptive response includes root architecture remodeling, ion transporter expression changes, and metabolic reprogramming (Munns et al., 2016). The new leaves that grow in salty conditions tend to have better tolerance due to anatomical changes such as increased succulence and altered ion distribution patterns (Flowers &Colmer, 2008). Recovery capacity differs between species and

genotypes, with some species having rapid growth resume and others having permanent damage after stress relief, these timely dynamics are important for the design of experiments because functional validation of transporters needs to take into account their contributions (Munns et al., 2006).

2.2.3 Signaling Pathways and Membrane Transport Proteins during Salt Stress

The membrane transport proteins play a crucial role in salinity tolerance in plants as they directly regulate the cellular and organelle ion flux. For plants under saline conditions, it is close to impossible to avoid high cytosolic Na^+ levels without the activity of specific transporters and channels maintaining essential K^+ nutrition. (Munns & Tester, 2008) These proteins play three important roles: (i) Extruding Na^+ from the cytosol into the soil or apoplast for removing Na^+. (ii) Separating Na^+ into the vacuole, thus preventing metabolic disturbance. (iii) Transporting Na^+ back into the cytosol from the xylem sap, to reduce the influx of Na^+ to the photosynthesizing tissues of the plant (Zhu, 2003; Blumwald, 2000). Understanding this, you can see how with properly functioning membrane transporters even relatively high soil salinity can cause massive imbalance and the death of cells. Identifying, regulating, and comprehension of the biochemical mechanism of these proteins are therefore crucial for crop genetic engineering to achieve salt tolerance. Some notable families are the SOS1 Na^+/H^+ antiporters (Shi et al., 2000), the *NHX* vacuolar exchangers (Apse et al., 1999), and the HKT family of sodium and potassium transporters (Ren et al., 2005). These key players, namely AtHKT1;1, which was the focus of this research are described in the following sections with special emphasis on AtHKT1;1.

Calcium signaling and the SOS pathway are the best-understood signaling cascades in plant salt responses (Zhu, 2002). The rapid rises in cytosolic Ca^{2+} concentration within seconds to minutes of exposure are caused by salt stress. This calcium signal is sensed by SOS3 (a calcium-binding protein), which activates SOS2 (a serine/threonine protein kinase). The SOS3-SOS2 complex then phosphorylates and activates SOS1, a plasma membrane Na^+/H^+ antiporter that extrudes Na^+ out of the cytoplasm (Qiu et al., 2002). The pathway demonstrates how plants recognize physical stress and utilize particular transport responses in response to these perceptions through coordinated signaling cascades.

The stress response to salt is integrated with larger developmental and environmental indicators through hormonal regulation. The key hormone in responses to osmotic stress is abscisic acid (ABA), which accumulates rapidly under salinity conditions and triggers stomatal closure, expression of stress responsive genes, and metabolic modification (Cutler et al., 2010). Ethylene is also a contributor to senescence responses, and it regulates growth in response to stress (Tao et al., 2015). Also involved in salt stress signaling are jasmonates and salicylic acid, which are mainly associated with biotic stresses but also play a role in salt stress signaling via complex cross-talk with ABA pathways (Yang et al., 2019). Brassinosteroids have been shown to increase stress tolerance by several mechanisms such as increased antioxidant capacity and altered expression of ion transporters (Divi & Krishna, 2009).

2.3 Membrane Transport Systems in Ion Homeostasis

2.3.1 Potassium Homeostasis Under Salinity.

Mechanisms to keep K^+/Na^+ selective are vital to the concept of salt tolerance because for many cellular functions K^+ plays a positive role, whereas Na^+ at similar concentrations is toxic (Munns & Tester, 2008). There are several strategies plants use to adapt to saline environments in which Na^+ predominates in the medium so that they can maintain K^+ uptake and transport. High affinity K^+ transporters are able to take up K^+ despite allowing much higher concentrations of Na^+ (Gierth&Mäser, 2007). The voltage dependent inward-rectifying K^+ channels open with a hyperpolarized membrane potential and conditions that also facilitate Na^+ uptake, and consequently involve other selectivity mechanisms (Véry&Sentenac, 2003). A major feature of plant salt tolerance is the coordination of excretion of Na from the plasma coupled to the uptake of K^+ under normal and stress situations, AKT/KA channel and their regulation gets involved in the uptake and distribution of K^+. These channels, which are Shaker type, regulate voltage dependent fluxes of K^+ across plasma membrane in different tissue in the context of which they are expressed in a particular way (Very &Sentenac, 2003). The activity of the channels is regulated by phosphorylation, interaction with regulatory subunits and their membrane potential, which enables strong regulation of K^+ fluxes according to the needs of the cells and the environment (Chérel et al., 2014). Under salinity, it is important to ensure the function of AKT/KAT channels to ensure K^+ nutrition and reduce the uptake of Na^+ through competing channels.The regulation of multiple transport systems is required in order for coordination to take place between K^+ uptake and Na^+ exclusion. As Na^+ enters the cell, outward-rectifying K^+ channels are activated, causing loss of K^+ further reducing the K^+/Na^+ ratio (Shabala et al., 2006). Rapid activation of H^+-ATPases to restore membrane potential after the loss of K^+, plus greater Na^+ extrusion by SOS1 and Na^+ sequestration by vacuolar NHX transporters (Zhu, 2003) will result in the prevention of this K^+ loss. This coordination underscores the interrelatedness of different systems of ion transport and the need to coordinate salt tolerance and ion regulation in understanding and engineering salt tolerance.

2.3.2 High-Affinity Potassium Transporter (HKT) Family

Discovery and Historical Context

A cDNA clone that was found from wheat (*Triticum aestivum*) roots, after isolation in a yeast mutant defective in K^+ uptake, was the founding member of the HKT family (Schachtman& Schroeder, 1994). This gene, designated as HKT1, was found to confer high affinity K^+ uptake to the yeast host and the transporter was shown to have the substrate's affinity, and cation selectivity indicating classical high affinity K^+ uptake in plants. The soluble protein was found to be a membrane protein involved in co-uptake of K^+ and H^+, and was expressed in specific regions of roots and leaves, which are the major organs involved in K^+ uptake.

Origin of the Name and Functional Reinterpretation

The name HKT is derived from the reflectivity on the high affinity of the protein for K^+ by mediation of the high affinity K^+ uptake in heterologous systems, for which the protein was originally assigned. Soon, though, other studies showed other cations as well, in particular Na^+, can be transported by one of the three types of HKT proteins. The functional versatility prompted the identification that HKTs were involved in K^+ nutrition and Na^+ homeostasis, especially in plant salinity tolerance. The family is designed around a common structure (Durell et al., 1999) and this is shared with the other families Trk/Ktr/HKT of cation transporters.

Structural Architecture

The unique feature of the HKT transporters is a tight packing of four strictly repeating segments, called membrane-pore-membrane (MPM) modules, each of which has two flanking transmembrane segments followed by a pore loop. This arrangement gives rise to a protein containing eight transmembrane helices and four pore loops (P loops) located in the center of the lipid membrane (Uozumi et al., 2001). The highly characterized plant HKTs form a selectivity filter of four glycines that are located in the four pore loops (Mäser et al., 2002).The membrane topology of HKT1 (AtHKT1;1) from *Arabidopsis thaliana* was carefully explored with a series of gene fusions with alkaline phosphatase in *Escherichia coli*, glycosylation scanners in microsomes and immunocytochemistry in human epithelial (*HEK293)* cells. These experiments have conclusively shown *AtHKT1;1* has eight transmembrane spanning segments, with both the N-term and C-term in the cytosol, and with the pore forming loops partially exposed to the outside (Uozumi et al., 2001). The general fold of HKT proteins is similar to a dimer formed by four MPM subunits. These two high-resolution crystal structures have recently been determined: Wang et al. (2023) determined the crystal structure of AtHKT1;1 in NaCl at 2.7 Å resolution.

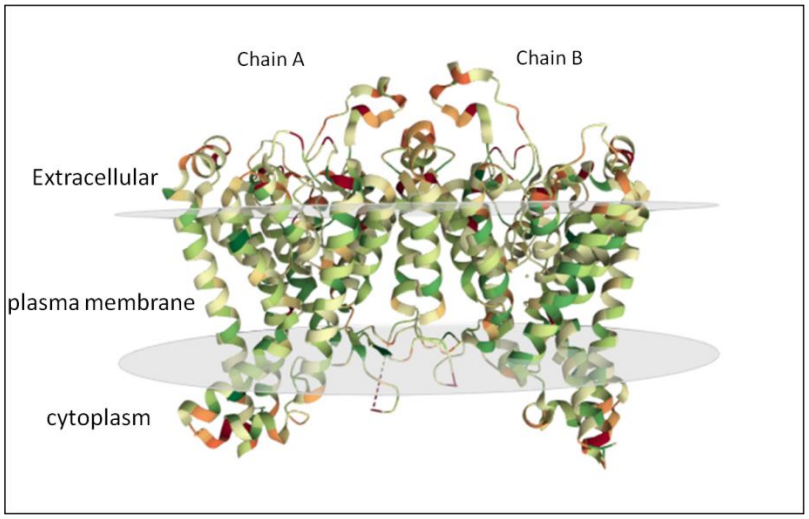

Fig:3.Structure of AtHKT1;1 in NaCl at 2.7 Angstroms resolution. (PDBID: 8W9N)

Evolution from Simple K^+ Channels

The history of the HKT transporters suggests that they have been obtained by multiple steps of gene duplication followed by fusion of four original MPM modules into a single polypeptide, starting from simple K^+ channels of the KcsA type. The Trk/Ktr/HKT family is then an evolutionary stage between the tetrameric K^+ channel (four separate subunits) and the unchained protein with four internal channel like domains (Durell et al., 1999). Both the presence of a K^+ channel. like selectivity filter in each pore loop and the cation selective properties that define the family arises from this evolutionary history.The assignment of FAMAS categories and markers of functional specialization. Assignment to FAMAS categories, markers of functional specialization, based on the single amino polymorphism in the primary pore loop, the HKT family can be subdivided into two major classes (also called subfamilies 1 and 2) (Mäser et al., 2002).In the first of the two selectivity filter motifs, class I (subfamily 1) members have the sequence **S**GGG. These proteins primarily act as Na^+ selective uniporters and are the key mechanism for retrieval of Na^+ in xylem of plants that are salt tolerant. Most class I HKTs are located in the root, in either root/stellar cells or xylem parenchyma and are responsible for excluding Na^+ from the xylem sap before it reaches the shoots, protecting the shoots from salt damage (Davenport et al., 2007). AtHKT1; 1 is one of the prototype of the class I.

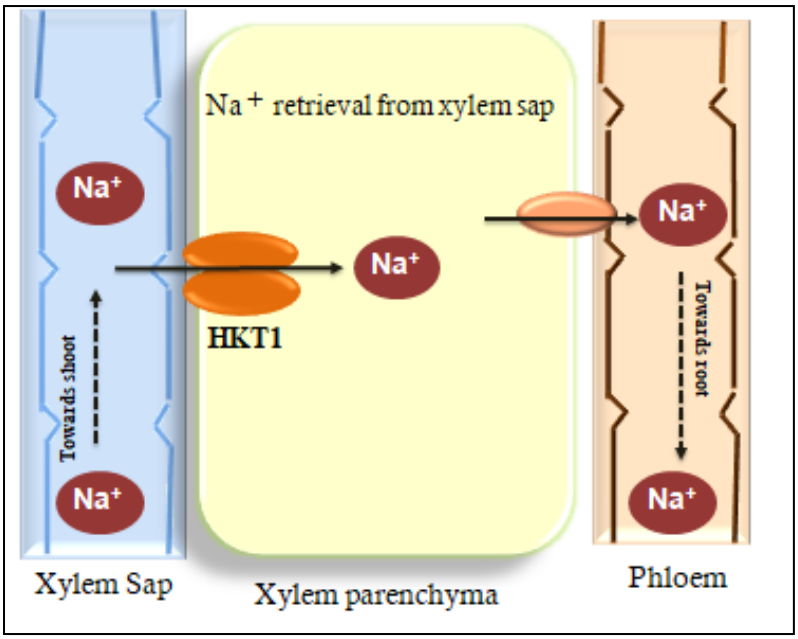

Fig. 4: Model showing AtHKT1;1 localization and function in the root

Class II (subfamily 2) members have a glycine residue (**G**GGG) at the same site. These proteins can mediate coupled Na^+-K^+ transport, and can also facilitate Na^+-K^+ symport. The expression of these is frequently associated with the ability to take up K^+ under conditions of K^+ deficiency, but also leads to higher Na^+ influx under saline conditions (Mäser et al., 2002).

There are nine major phylogenetic types of the HKT family in angiosperms, with especially high gene duplication events provided by duplication in the HKT1; 5 and HKT1; 4 cluster that resulted functional divergence between similar isoforms (Frontiers in Plant Science, 2025). To date, 25 co-evolved residue groups, which we believe is important for the stability and integrity of the function of HKT proteins, have been identified through analysis of co-evolution (Frontiers in Plant Science, 2025).

Physiological Functions

The primary function of HK transporters in plant salinity tolerance is through a Na^+ retrieval mechanism in the vasculature. *Arabidopsis* expression patterns indicate that AtHKT1;1 is present in the xylem parenchyma of the root, and in the phloem of the stamen, where it traps Na^+ in the xylem sap and brings it back into the roots, preventing excessive Na^+ build-up in leaves and maintaining reproductive fertility under salt stress (*Arabidopsis*) (Davenport et al., 2007; Uchiyama et al., 2023). Other member of class I of *OsHKT1;5*(Osaka et al., 2005) play a similar role in the rice plant and variation in these genes is a primary factor that contributes to the intra-specific variation in salt tolerance. The Kna1 locus responsible for shoot Na^+ exclusion was found to be associated with a HKT1;5 allele in wheat and favorable alleles have been incorporated into commercial varieties to enhance saline wheat yields (Byrt et al., 2014).A second, K^+ dependent function, carried out by class II HKTs, can in some species result in Na^+-K^+ co transport in certain species and thus also contribute to the K^+-up uptake in K^+ deficiency. At high Na^+ levels other transporters may also aid in the uptake of Na^+ and their net contribution is more complex and species dependent (Mäser et al., 2002).The HKT family provides a prime example of how the process of gene duplication, sequence evolution and differential regulation of expression can generate a flexible toolkit of cation transporters which plants trigger to allow them to maintain their ion homeostasis under different environmental salinities.

2.4 Challenges in Plant Membrane Protein Biochemistry

2.4.1 Technical Limitations

Low abundance of native transporters in plant tissues is one of the major limitations for biochemical and structural characterization (Møller et al., 2008). For most applications, purification from native tissues (nervous tissue for instance) is impractical because the most important transporters form less than 0.01% of membrane protein load. (Rosenbusch, 2001). This means biochemical studies are resource intensive, requiring either large scale plant growth and/or complex purification techniques, and are often difficult to execute and/or costly. Purification is often unsuccessful, and when it is, yields are generally too small to be characterized in detail or for structure determination studies that require about 1 mg of protein (Bill et al., 2011).These proteins are challenging to work with due to stability problems when released from membranes. Most, if not all, of the structural integrity and function is lost within hours or minutes of being removed from the native lipid environment (Loll, 2003). Solubilization with detergents results in purified protein with activity although this is vital for purification, it

may be seen to disrupt essential interactions between the protein and lipids that are important for activity and stability. Extension and empirical optimization of conditions that maintain protein function during extraction, purification and subsequent manipulation, is required for each new target protein (Carpenter et al., 2008).

The Lipid environment reflects in turn the close association of the lipids with membrane proteins. The proper folding, stability, or activity of specific lipids might be required, and other lipids (such as phospholipids), sterols, and other components of the membrane can modulate the function of transporters by direct binding or by altering the physical properties of the membranes (Lee, 2004). Proteins must be reconstituted into a defined lipid environment to perform mechanistic studies, but it is possible that this reconstitution could induce changes in the behavior of proteins. These challenges are now starting to be addressed by recent development of nanodisc technology and lipidomic analysis (Denisov &Sligar, 2016).In the past, however, it has been hard to investigate plant transporters in detail with crystallographic techniques due to their difficulties. Crystallization of membrane proteins is extremely difficult due to their hydrophobic surface, inherent flexibility and dynamics and a strong need to be stabilized with detergents (Caffrey, 2009). Despite its success, cryo-electron microscopy (cryo-EM) of membrane proteins has not yet become an easy structural biology approach and many transporters from plants have resisted structural determination (Cheng, 2018).

2.4.2 Expression Systems Comparison

A good heterologous expression system is essential for studying the plant membrane protein AtHKT1;1. Various systems were tested and *Pichia pastoris* was used to perform the functional validation of this Na^+ pump, these are:

Escherichia coli

E. coli is the 'easiest' system due to its high productivity (10–100 mg/L), low cost and ability to grow quickly, making it the most convenient of all systems (Junge et al., 2008). Owing to its large gene reservoir, a range of engineering approaches can be taken (Makino et al., 2011).Restrictions: the machinery of post-translational modifications and/or the machinery required for the proper folding of eukaryotic membrane proteins (Grisshammer& Tate, 1995). Multi-transmembrane proteins, such as AtHKT1;1 (8 predicted TM helices) are often observed as inclusion bodies (Baneyx&Mujacic, 2004). The rate of success in refold is low. Thus, *E. coli* was decided against as a functional source of AtHKT1;1.

Insect Cells (Sf9/Baculovirus)

Advantages: is excellent folding and authentic post-translational modifications that are closely similar to those of plant cells (Jarvis, 2009).

Restrictions: It is expensive and has low yield (0.1-1mg/L) and the protocol is complicated. It takes weeks longer to obtain the recombinant baculovirus (Portolano et al., 2014). For the simple and frequent requirement of functional validation of AtHKT1;1, this system is too expensive and not high throughput.

Mammalian Cells

The advantages include a native environment, lipids and modifications (Andrell& Tate, 2013). Restrictions are the high cost, ethical issues and the yield ranges in micrograms rather than milligram levels (Almo and Love, 2014). Feasible on a much smaller scale than needed in this project.

Saccharomyces cerevisiae

Advantages: eukaryotic processing and insertion into membranes and simple post-translational modifications (Daly & Hearn, 2005). Functional complementation assays are possible using well-characterized mutants that are salt sensitive. (Quintero et al., 2002).

Restrictions: Expression of the AtHKT1;1 in *S. cerevisiae* is often to internal membranes, making it difficult to study its function. This method is quite nice as a complementary system but is not able to provide enough milligram amounts of a compound for biochemical validation.

***Pichia pastoris (Komagataellaphaffii)*: Selected System**

Advantages: The AOX1 promoter will achieve very high levels of expression; with AOX1 expression achieving up to 30% of total cellular proteins (Cereghino&Cregg, 2000). High cell density fermentation produces 10-100x more protein than shake flasks. E.g. less heterogeneous, smaller m mannose-chains than in *S.cerevisiae* (Bretthauer&Castellino, 1999). A variety of plant membrane proteins have been functionally expressed in *Pichia* such as ion channels, aquaporins and H^+-ATPases (Villalba et al., 2005; Nyblom et al., 2007). With *Pichia,* AtHKT1;1 has the most favorable combination of eukaryotic folding and high yield to be further analyzed by SDS-PAGE, Western blot and functional assays.

Restrictions: Methanol is toxic at > 0.5–1%, and requires fed-batch control (Macauley-Patrick et al., 2005). Genes are prone to deletion from multicopy integration strains with long-term cultivation (Zhu et al., 2009). Proteolytic degradations can happen but strains that are protease deficient (e.g., SMD1163) will work. These restrictions can be overcome through optimization of the processes.

Conclusion of Comparison

Pichia pastoris offers an ideal combination of eukaryotic processing, high yields, low glycosylation and cost-effectiveness for functional validation of AtHKT1;1which requires detection of protein by SDS-PAGE and Western blot and a measurable ionic phenotype in a live host. It is known to be successful working with plant transporters and is appropriate to this project with its objectives.

2.5 *Pichia pastoris*: A Versatile Platform for Membrane Protein Production

2.5.1 System Development and Optimization

P. pastoris evolved into a leading heterologous expression host for producing proteins starting from its use as methylotrophic yeast in the production of single cell industrial protein (Cereghino&Cregg, 2000). The system was originally designed in the 1970s for single-cell

protein (SCP) production using methanol as input, but after the oil crisis in the 1980s which demanded the production of SCP to be uneconomical, the system was repurposed for the production of recombinant protein in the 1980s (Cregg et al., 1993). High cell density fermentation capacity combined with powerful promoters with strong and easily adjustable activity levels resulted in an extraordinarily efficient expression system that has been ongoing improved over the past 40 years (Macauley·Patrick et al., 2005).The choices available include X-33, GS115, KM71H characteristics, with strain development (Cregg et al., 2000). The commonly used laboratory strain, GS115 is His autotrophic (his4) which allows for selection of transformants that are able to complement this mutation. KM71H contains a mutation in the AOX1 gene to give slow methanol utilization which may be beneficial to some proteins due to slower methanol consumption rate (Stratton et al., 1998). X-33 is the wild type parent strain with intact AOX1 (Mut^+ phenotype) and no autotrophies, suitable for applications where selection markers other than HIS4 are used (Invitrogen, 2010).Flexible expression strategy for vector systems: secretion versus intracellular (Cereghino&Cregg, 2000). The pPICZ series, such as pPICZ-A employed here, is based on the AOX1 promoter, which is able to be induced by methanol, and contains the α-mating factor secretion signal of *S. cerevisiae*.

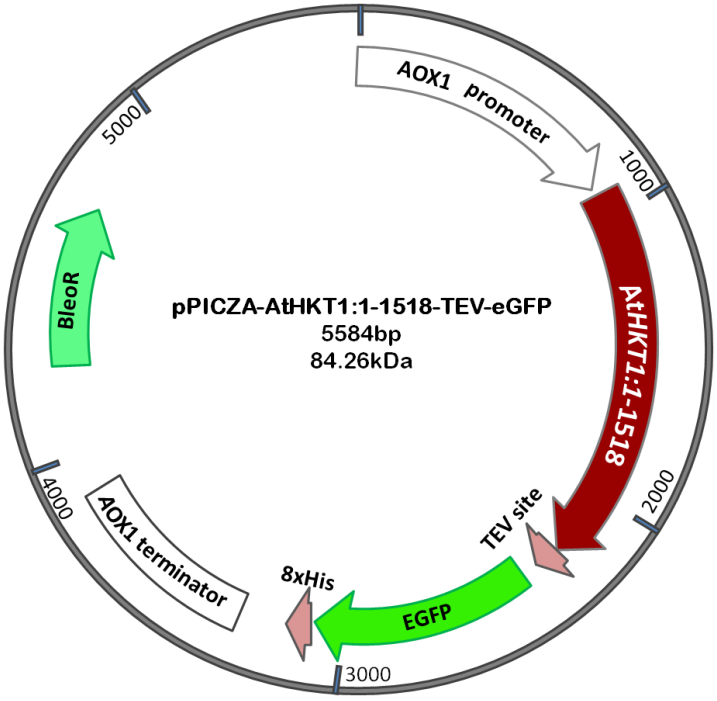

Fig.5: Schematic representation of the expression cassette used for the heterologous expression of AtHKT1;1 in *Pichia pastoris*

A special expression cassette was prepared and inserted into the pPICZ-A vector for this study. The AOX1 promoter, the AtHKT1;1 coding sequence, the TEV protease cleavage site, the EGFP (enhanced green fluorescent protein) tag and the 8×His tag are present in the cassette from the N-terminus to the C-terminus. The TEV site is used for removal of the EGFP tag when desired,

the EGFP tag provides visualization of the localization of the protein and the 8×His tag allows the protein to be purified by immobilised metal affinity chromatography (IMAC).

2.5.2 SMALP Technology for the extraction of membrane proteins

Removal from the native lipid bilayer during extraction and purification may result in loss of structural integrity, decreased stability and decreased activity (Lee, 2004; Loll, 2003). So it is critical to maintain the native lipid environment of the membranes during the extraction process to preserve the functionally active membrane proteins in *Pichia pastoris*.Styrene-maleic acid (SMA) copolymers offer a powerful alternative to conventional detergents for membrane protein extraction. SMA copolymers spontaneously enter the lipid bilayer and disrupt them into nanoscale, disc-like particles (SMALPs) that consist of the membrane protein, surrounded by a native lipid belt (Dörr et al., 2016; Jamshad et al., 2014). This is a method where the protein-lipid interactions important for stability and function are preserved, whereas the detergent-based method removes lipids around the protein.For the extraction of a variety of transporters and ion channels, a copolymer of styrene and maleic acid (SMA2000) has been found to be particularly effective (Lee et al., 2019). When added to membranes, it not only solubilizes protein-lipid complexes, but also preserves the protein in near native state. SMA2000 extraction is generally carried out between a pH range of 8.0, at room temperature for a period of 1-3 hours, depending on the membrane type and the protein of interest.

For any particular protein, it is advisable to conduct systematic small-scale screening to find the best extraction conditions before scale-up (Pollock et al., 2018) showed that extraction efficiency could vary significantly depending on various parameters like pH, temperature, incubation time, and SMA concentration, among others. The supernatant and pellet fractions can then be analyzed by western blot to quickly determine conditions for achieving maximum protein recovery with protein integrity. This empirical optimization approach was followed in this study to ensure efficient solubilization of AtHKT1;1 from *Pichia pastoris* membranes.(Dutta et al., 2020)There are a number of benefits to using the SMALP approach instead of detergents. The native lipid environment, protein-protein and protein-lipid interactions, and the ability of a SMALP to be used for a variety of downstream applications such as mass spectrometry, electron microscopy, and functional assays (Denisov &Sligar, 2016). Furthermore, the preparation for SMA does not need the exogenous lipid to be added during purification process, which makes the process easy and simpler to be done and leads to less variability.For this study, SMA 2000 was used to solubilise*AtHKT1;1* from the *Pichia pastoris* membrane fractions. Large scale extraction was done after initial small scale screening to determine the best pH, temperature and incubation time for extraction, and then subjected to nickel affinity chromatography and biochemical characterization.

2.5.3 Membrane Protein Specific Considerations

The choice of tag for purification (8xHis-tag, eGFP) affects purification efficiency and behavior of the protein (Terpe, 2003). For this study, apolyhistidine tag (8x His) is employed to allow

immobilized metal affinity chromatography (IMAC) and SMA (Hochuli et al., 1988) extraction. EGFP fusions can be used for visualization during optimization of the expressed protein as well as localization studies but interfere with functionality and the protein has to be cleaved for functional studies (Drew et al., 2005). Cleavable tags based upon TEV protease are used after purification to remove the tag for applications where the native protein is required (Waugh, 2011).Membrane proteins often exhibit abnormal behavior on SDS-PAGE. The tendency of the migration of hydrophobic membrane proteins to migrate at apparent molecular weight that is very different from its calculated molecular weight is well documented (Rath et al., 2009). Incomplete denaturation, secondary structure and the presence of hydrophobic transmembrane domains, which bind SDS differently from soluble proteins, is thought to be responsible for this anomalous migration. Consequently, a membrane protein can be displayed as being larger or smaller than its theoretical molecular weight on SDS-PAGE and size determination by SDS-PAGE is therefore not sufficient to determine the molecular weight.

2.5.4 Case Studies Relevant to my Project

A very successful expression of membrane proteins in this system is the expression of H^+-PPase from the plant *Pichia*. The AOX1 promoter was used to express *Arabidopsis* vacuolar H+-pyrophosphatase (AVP1) in GS115 at a concentration of 0.5 mg/L. The expressed protein proved to retain activity in membrane vesicles (functional assay) with similar kinetics to the native protein. Key findings: pH dependence, inhibitor sensitivity indicate that the protein expressed in *Pichia* recaptures properties that were seen in plant membranes, making them a good platform for mechanistic studies(Costa et al., 2018).*Pichia* is used for localization and transport studies as exemplified by *Arabidopsis* sugar transporter (Zhang et al., 2020). Proper folding and processing of the fusion was evidenced by the correct targeting of GFP to the plasma membrane in *P. pastoris* through localization studies. Radioactive sugar assays were used to determine the kinetic parameters and the sugars specificities allowing sugar allocation in stress conditions.*Pichia* is useful for functional complementation and electrophysiological characterization as illustrated with rice ammonium transporter (Chen et al., 2019). In yeast mutants, growth complementation was demonstrated and it was shown that the plant transporter could replace their endogenous ammonium uptake systems. Enzymes and transports mechanism and regulation were characterized by electrophysiology by using solid-supported membranes, offering insights into nitrogen nutrition under stress.

2.6 Knowledge Gaps and Research Questions

2.6.1 Specific Gaps in Understanding

No attempt has been made to express, purify and/or isolate the AtHKT1;1 protein in the *Pichia pastoris* expression system. Other systems for obtaining plant membrane transporters such as *Apiatumhispanum* (Villalba et al., 2005) or A. donax (Nyblom et al., 2007) have shown good results, however there has been no published report on expression, extraction, and purification of AtHKT1;1 from *P. pastoris.* As a result, basic information is not known, including the optimal conditions of induction, the selection of strains, the membranes yield and content of extraction in

SMA2000. However, lack of an established purification pipeline makes an access to pure protein for mechanistic studies difficult.This project will ultimately yield information that will facilitate further research of the protein and lipid interactome of AtHKT1;1. The successful validation and purification of an active AtHKT1;1will provide a basis for the identification of membrane-associated proteins and lipids linked to AtHKT1;1 in its native environment. Future studies, using SMA2000 extraction and mass spectrometry, could be used to characterize the lipid composition of the lipid nanodiscs that are associated with AtHKT1;1, and the identification of interacting partners that regulate the transport activity of AtHKT1;1.

2.6.2 Technical Limitations Addressed by This Study

The yield from expression and detection of AtHKT1;1 in *Pichia pastoris* is yet to be determined. Many plant membrane proteins (Popova et al., 2023) have been successfully expressed in *Pichia*, but there is no published report on the expression and observation of AtHKT1;1 in this host. There is no report of extraction from this protein class using SMA2000. While successful for membrane proteins as native lipid bilayer nanodiscs (Dörr et al., 2016; Lee et al., 2019) the application of SMA2000 to plant HKT transporters is yet to be tested. The optimal parameters for a maximum amount of optimally functional AtHKT1;1 recovery from *Pichia* membranes are determined experimentally, such as buffer composition, pH, incubation times and temperatures.

CHAPTER 3

Research Gaps and Objective

3.1 Research Gaps

Although AtHKT1;1 is known to play a crucial function in the salt tolerance of *Arabidopsis*, one important knowledge gaps exists:

AtHKT1;1expression, isolation and purification in *Pichia pastoris* has not been attempted.

Although the function of AtHKT1;1 was confirmed in planta and Xenopus oocytes (Sunarpi et al., 2005; Uozumi et al., 2001), no report has been done regarding the ability of the protein to localize correctly to the plasma membrane in *Pichia pastoris* cells. While several plant membrane proteins such as AGP and Hevein have been successfully expressed and purified from *Pichia pastoris* (Villalba et al., 2005), AtHKT1;1 has not. Optimal induction, strain selection, and SMA2000 extraction protocols for this transporter are lacking. This thesis addresses these gaps, providing a basis for future interactome and structural characterization.

3.2 Research Objective

To solve the identified gaps, this study has two major objectives, which are;

Objective 1: Overexpressionof AtHKT1;1 in *Pichia pastoris.*

This includes overexpression ofthe transformedcells, optimization of induction conditions and verification of expression on SDS-PAGE and Western blot.

Objective 2: Purify and isolate AtHKT1;1 over expressed *in Pichia pastoris;*

Membrane fractions will be extracted using SMA2000 (which preserves native lipid nanodiscs), followed by IMAC purification via the 8x His-tag. Success will be assessed by SDS-PAGE and Western blot.

CHAPTER 4

Material and Methods

4.1 Yeast Strains and Culture Conditions

AtHKT1;1 was heterologously expressed in the methylotrophic yeast *Pichia pastoris* (now called *Komagataellaphaffii*) strain GS115. It has an intact alcohol oxidase 1 gene (AOX1) and thus matches the Mut^+ (methanol utilization positive) phenotype and can grow normally using methanol as the sole carbon source. To use this trait for high level expression of a gene, tightly controlled by a methanol inducible AOX1 promoter, was exploited.GS115 was maintained on routine sub-culturing on Yeast Peptone Dextrose (YPD) agar plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) at 30 °C for 2–3 days. Plates were kept at 4 °C for up to 14 days.

4.2 Media Preparation, Plating, and Culture Initiation

4.2.1 Preparation of YPD Media

The yeast peptone Dextrose (YPD) medium was prepared to a total of 100 mL that consisted of 1 % (w/v) yeast extract (1 g), 2% (w/v) peptone (2 g), and 2% (w/v) D-glucose (2.5 g; a slight excess was added to compensate for potential degradation during autoclaving).The dissolution is done in deionised water and the volume made up to 100 mL. The solution was then divided equally into two 250 mL Erlenmeyer flasks (approximately 50 mL each). 2.0 g agar was added to 2 of the flasks for making Culture plates. The solutions were sterilized by autoclave at 121 °C for 15 min all flasks.

4.2.2 Addition of Antibiotics and Preparation of Selective Plates

Antibiotics were added directly into molten media as required by the experiments, after autoclaving the media has been cooled down to about 55 °C.

- **For transformed cells (AtHKT1;1-expressing)**: Zeocin (150 μg/mL) and kanamycin (50 μg/mL) were added to one of the flasks containing agar. The medium was then poured in sterile Petri dishes and dried.
- **For wild-type cells**:To the second flask, Kanamycin (50 μg/mL) was added. The medium was poured into sterile Petri dishes, and then solidified.

4.2.3 Streaking and Incubation

Both the transformed cells (with the pPICZ-A-*athkt1;1*-His construct) and the wild-type cells were streaked with a sterile inoculation loop onto the respective selective plates. Transformed cells were added to the plate with both Zeocin and kanamycin; while the wildtype cells were added to the plate with kanamycin only. All plates were parafilmsealed and then placed

meanwhile upside down at 30 °C for upto 3 days for the appearance of separate colonies. Plates containing colonies were kept at 4 °C and subsequently used for obtaining cells for all experiments.

4.3 Culturing in BMGY medium.

4.3.1 Preparation of BMGY Medium

Standard Pichia expression protocol was used to prepare Buffered Glycerol-complex Medium (BMGY). The yeast extract in the medium was 1%, the peptone 2%, the potassium phosphate buffer 100mM, the yeast nitrogen base (YNB) 1.34%, biotin $4\times10^{-5}\%$ and glycerol 1%. 250 mL of BMGY was prepared and autoclaved, and then left to cool to room temperature.

4.3.2 Addition of Supplements and Antibiotics

Antibiotics and biotin were added to the BMGY medium under sterile conditions just before the inoculation in a laminar flow cabinet. All 250 mL of BMGY was supplemented with the following components:

- Kanamycin (final concentration of 50 µg/mL)
- Biotin (0.4 µL/mL)

The supplemented BMGY was then split in half evenly, into two sterile 1 L conical flasks labeled A and B having 125 mL of medium each:

- Transformed cells: Zeocin at 150 µg/mL was added to a sample flask.
- Wild-type flask: No Zeocin (only kanamycin and biotin) was added to this flask.

4.3.3 Inoculation

Single colonies of transformed (AtHKT1;1 expressing) and wild type *P. pastoris* GS115 were picked from the selective plates with sterile tips. One colony was very carefully moved to the respective flask (transformed colony to the transformed flask, wild-type colony to the wild-type flask). The medium was gently vortexed with the tips, to break up cell clumping and promote growth.

4.3.4 Growth Conditions

The amount of shaking was 250 rpm, which happened in both flasks, and both flasks were then incubated overnight at 28 °C. The overnight cultures were then allowed to proceed to the next induction steps at an optical density of ~0.6-1 (diluted readings were taken using 100 µL sample and 900µL water) at 600 nm (OD_{600}) appropriate for further induction. The methanol-inducible cultures were directly used for the purposes of these cultures as described in Section 4.4.

4.4 Methanol Induction and Large-scale Expression

4.4.1 Preparation of BMMY Induction Medium

Buffered Methanol complex Medium (BMMY) was made with 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34% yeast nitrogen base, and 0.4µL/ml biotin. No Zeocin was added to the BMMY for either transformed or wild-type cultures. To avoid bacterial contamination, 50 µg/mL kanamycin was added to both cultures.

4.4.2 Harvesting and Washing of BMGY Cultures

The overnight BMGY culture was transferred from the incubator to the laminar flow cabinet. A small sample was removed to test for contamination and the optical density at 600 nm (OD_{600}) was measured, and found to be between 0.6 and 1(the OD was taken with diluted sample i.e. 100µl sample and 900µl d.H_2O). The culture was then placed in centrifuge bottles and centrifuged at 6000 rpm for 5 min at 25 °C. The supernatant was removed. The cell pellet was resuspended in autoclaved deionised water and then centrifuged for 5 min at 6000 rpm, at 25 °C. This water wash was repeated again (two washes). The wash water was removed after the last wash.

4.4.3 Resuspension in BMMY to Target OD

The washed cell pellet was resuspended in BMMY (supplemented with 50 µg/mL kanamycin and 0.4µl/mlbiotin) at OD_{600} upto OD 1. The volume was adjusted accordingly and the culture was then transferred to a sterile 1L baffled flask.

4.4.4 Methanol Induction & Daily Maintenance.

The flask was shaken at 250 rpm at 28–30 °C. To ensure induction, 0.5% (v/v) methanol was added every 24 h. Induction period = 72 h.Small samples were collected each day, before the methanol is added to the system, and checked by microscopy for morphological changes and contamination.

4.4.5 Anti-foam Addition

Foam was formed in the culture on the day after the induction started. The culture was supplemented with 5µL of anti-foam agent with to control foaming.

4.4.6 Cell Harvest after Induction

The culture was taken out of the incubator after 72 h and placed in the centrifuge bottles. The cells were harvested by centrifugation at 6000 rpm for 10 min at 25 °C. Supernatant was removed. The pellet was washed once with water and re-suspended directly in lysis buffer. This

ensured that the re-suspended pellet was stored at 4 °C until processing further (membrane isolation, Sections 4.5 and 4.6)

4.5 Direct Sonication for Membrane Isolation (Method A)

Direct Sonication is fast, easy and reproducible method of lysis and because of this reason it was used as the primary method of lysis in this study, without using enzymatic digestion of cell wall (Snijder et al., 2007). Pulsed Sonication was used to avoid the denaturation of the proteins and was conducted on ice. This method was selected because of its simplicity and compatibility with use with downstream membrane isolation and SMA2000 solubilization.The workflow is presented in Figure 6.

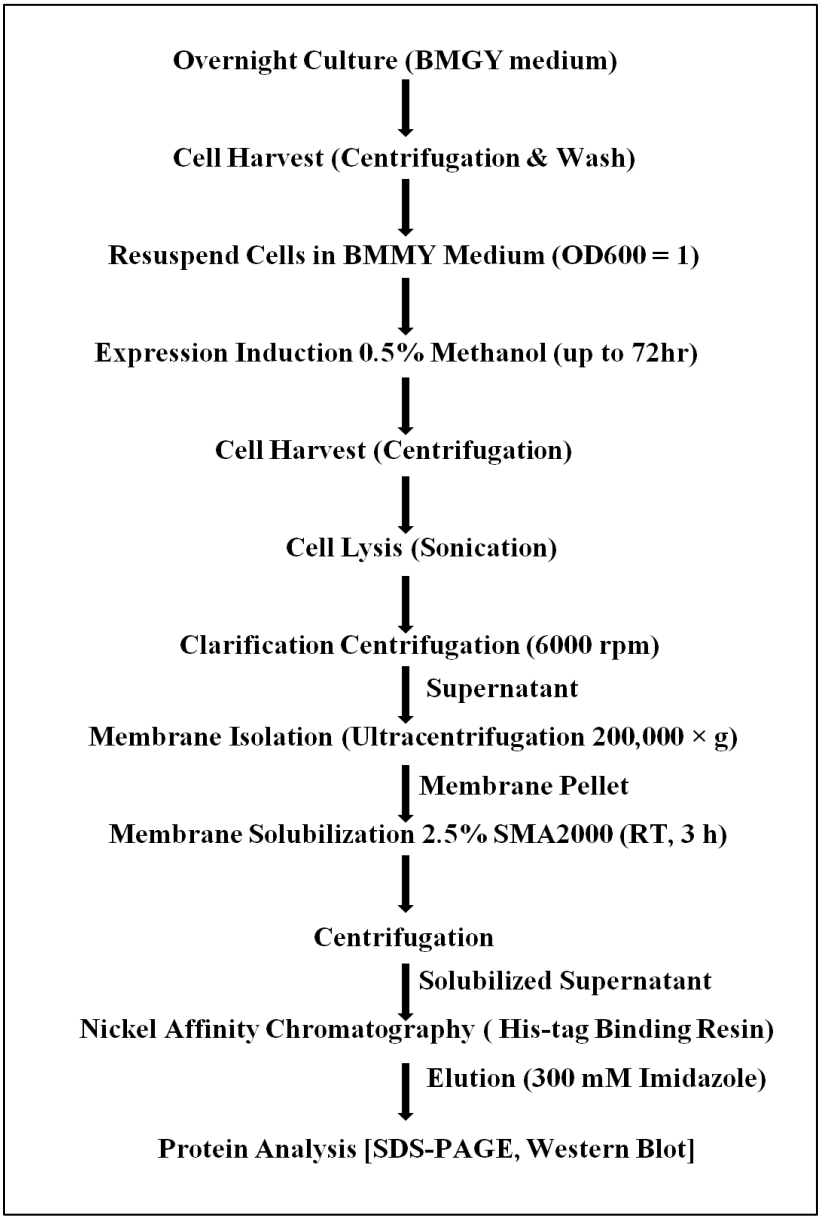

Fig.6: Experimental workflow for Direct Sonication for Membrane Isolation

4.5.1 Harvest and Lysis of Cells using Sonication.

The harvested cell pellet (from Section 4.4.6) was resuspended in hypotonic lysis buffer (20 mM Tris-HCl, pH 7.5, containing protease inhibitors). The suspension was transferred to a 50 mL Falcon tube and placed on ice. The Sonication was carried out with a probe Sonicated, with the following parameters: 10 ON seconds x 30 OFF seconds, amplitude 80% and with the run time for 30 min and 1mM PMSF is freshly added [protease inhibitor] to protect the proteins present during Sonication. The sample is observed under the microscope to check the cells morphology during and after Sonication.

4.5.2 Clarification Centrifugation

Unbroken cells and large debris were removed by centrifugation of lysate at 6000 rpm for 10 min at 4 °C. The supernatant (clarified lysate) was carefully removed and placed in a new tube and the pellet was discarded.

4.5.3 Membrane Isolation by Ultracentrifugation

The clarified lysate was ultra-centrifuged for 1 hour at 4 °C at 200,000×g (ultracentrifuge). The supernatant (cytosolic fraction) was discarded. The membrane pellet was carefully recovered and re-suspended in a small volume of resuspension buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl) by using gentle pipetting.

4.5.4 Solubilization with SMA2000

The membrane pellet was resuspended in SMA2000 buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl). Styrene-maleic acid copolymer (SMA2000) was added to a final concentration of 2.5% (w/v). The mixture was incubated at room temperature for 3 hours with shaking on a rotating mixer.The solubilized mixture was then centrifuged at 4 °C at 10,000×g for 30 min to remove the insoluble material. The supernatant (containing SMALPs loaded with *AtHKT1;1*) was transferred to a new tube with care.

4.5.5 Purification by Nickel Affinity Chromatography

The SMA2000-extracted supernatant was loaded onto a Ni^{2+}-NTA column pre-equilibrated with binding buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 30 mM imidazole). Non-specifically bound proteins were removed from the column by washing with 10 column volumes of wash buffer (binding buffer with 30 mM imidazole). Elution buffer was composed of 300 mM imidazole in the same base buffer with which the target protein (AtHKT1;1) was eluted. Fractions were collected and analysed for protein content (Section 4.7).

4.6 Membrane Isolation by the Zymolyase Mediated Spheroplast (Method B)

As an alternative method to mechanical lysis, Zymolyase mediated spheroplast method was employed. Spheroplasts can be lysed under hypotonic conditions with little mechanical stress, which limits the potential for protein denaturation and shearing (Hartmann et al., 2017). This method is especially useful for sensitive membrane proteins which could be unstable during Sonication. The method was evaluated alongside direct Sonication to compare protein yield, quality and to assess the best extraction protocol for AtHKT1;1 (Guyot et al., 2020). The workflow is presented in Figure 7.

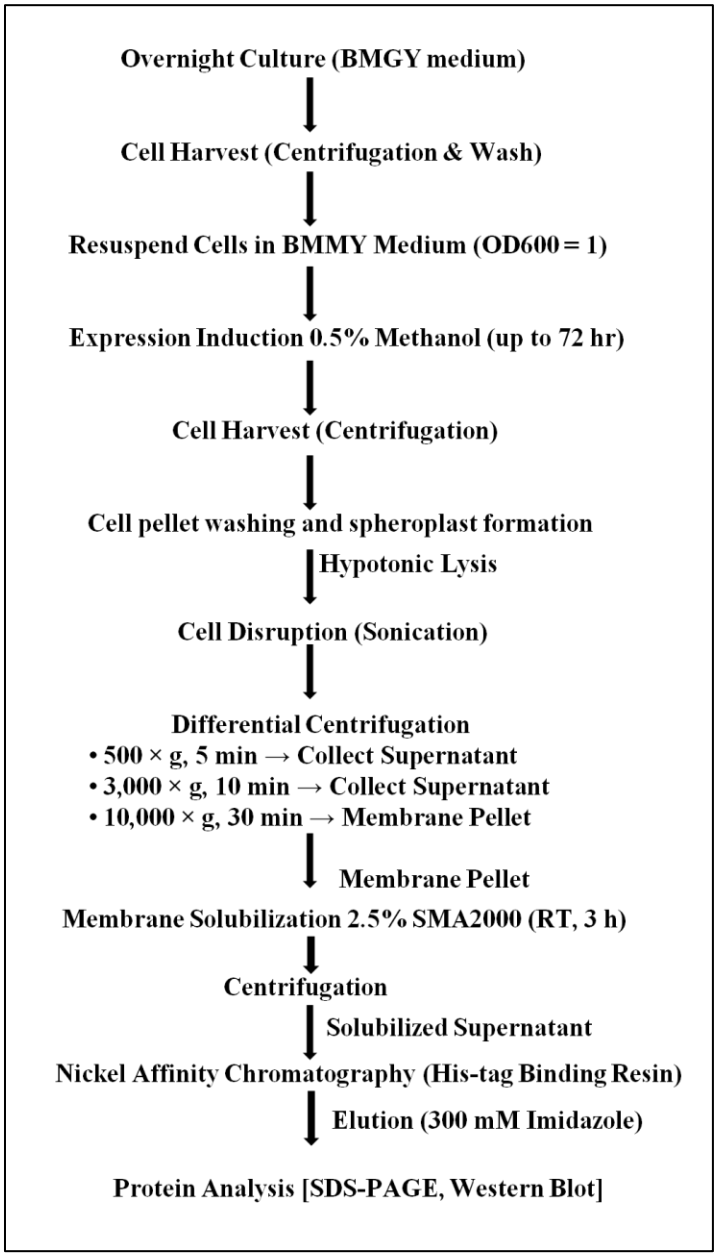

Fig.7: Experimental workflow for the Zymolyase Mediated Spheroplast

4.6.1 Cell Harvest and Preparation

The induced Pichia cells (from section 4.4) were collected by centrifugation at 5000 rpm for 3 min at 4 °C in 50 mL Falcon tubes. The liquid on top was poured off. The cell pellet was washed once with 20 mL of cold sterile water, then once with 20 mL of cold SED buffer (without β-mercaptoethanol).

4.6.2 Spheroplast

The washed pellet was then resuspended in β-mercaptoethanol + SED buffer (1 µL of β-mercaptoethanol / 100 µL of SED buffer). After adding the suspension and incubating at 4 °C for 5 minutes, 5 µL of zymolyase was added to each 50 µL of the suspension. The mixture was then incubated at 30 °C for 30-60 min with gentle shaking. The efficiency of spheroplast was evaluated by calculating the optical density of the cells at 800 nm before (T_0) and after (T_1) spheroplast formation:

% Spheroplast = 100 – [(OD_{800} at T_1 / OD_{800} at T_0) × 100]

A value >70% was considered acceptable. The suspension was subjected to efficient spheroplast formation and then centrifuged at 800×g for 5 min at room temperature and the supernatant was removed.

4.6.3 Washing and Cell Lysis by Sonication

The pellet of the spheroplast was washed twice in SED buffer. Buffer was added slowly to the wash tube each time, so as not to disturb the spheroplasts when they were added. The pellet was resuspended in 10 mL of the hypotonic lysis buffer, by slow pipetting (As we are using eGFP-tagged protein, a colour change was observed). The formation of spheroplasts was confirmed using a fluorescence microscope.The spheroplasts were then sonicated with a probe sonicator (5 s ON, 30 s OFF at 4 °C) for a total of 5 pulses. Lysis was confirmed by microscopy.

4.6.4 Differential Centrifugation for Membrane Isolation

The lysate was then centrifuged at 4 °C with a series of differential centrifugation steps:

- 500×g for 5 min – The pellet (unlysed cells, large cell wall debris) was discarded. The supernatant was kept.
- 3000×g for 10 min – The pellet (mitochondria, large organelle fragments) was discarded. Supernatant was kept.
- 10,000×g for 20 min – The supernatant was collected, and the membrane pellet was kept. Fluorescence was checked in both the supernatant and the pellet.

4.6.5 Solubilization with SMA2000

The membrane pellet was re-suspended in SMA2000 buffer (e.g., 20 mM Tris-HCl, pH 8, 150 mM NaCl). The optimal pH was checked before use (pH 8.0) for the SMA2000 solution. Suspension was shaken at room temperature for 2 h.

The mixture was then centrifuged at 10,000×g for 30 min at 4 °C to clarify. The supernatant (solubilized AtHKT1;1 in SMA2000-lipid particles) was carefully removed from the pellet into a new tube. The fluorescence was once again measured in both the supernatant and the pellet.

4.6.6 Purification by Nickel Affinity Chromatography

To eliminate the possibility of EDTA from interfering with nickel binding, the SMA2000 extracted supernatant (Section 4.6.5) was treated. A stock solution of 100 mM $NiCl_2$ was made for 10 mL total of sample and then 100 µL of this stock was pipette into the sample. The mixture was kept on ice for 5 min to ensure excess Ni^{2+} ions bind to EDTA. After this incubation the sample was applied to a Ni^{2+}-NTA column pre-equilibrated with binding buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 30 mM imidazole). The unbound proteins were removed by washing the column with 10 column volumes of wash buffer (binding buffer with 30 mM imidazole). The elution buffer used was 300 mM imidazole in the same base buffer for elution of the target protein (AtHKT1;1-His). The protein content of fractions was determined as per Section (4.7).

4.7 Protein Detection and Analysis (for both methods)

4.7.1 Sample Preparation for SDS-PAGE

Protein samples obtained during the different purification steps were combined with 2× loading dye in the ratio of 80 µL protein to 20 µL 2× loading dye. The loading dye was prepared with 1 mL of 1 M Tris-HCl (pH 7.5), 0.4 g SDS, 2 mL glycerol, 2 mg of bromophenol blue, 1 mL of β-mercaptoethanol and water to a final volume of 10 mL. The *Pichia* protein samples were not heated before loading as is done for bacterial samples. Samples were then mixed and briefly vortexed and loaded directly onto the gel.

4.7.2 Gel Preparation and Electrophoresis

In each set of samples 4 SDS-PAGE gels were made. The Coomassie staining was done with two gels (Gel 1 and Gel 2), while the western blotting was performed with two gels (Gel 3 and Gel 4). The separating gel was always 10% and the stacking gel was always 4-5%.The separating gel (10 mL total volume) was prepared from 4.1 mL water, 2.5 mL of 1.5 M Tris-HCl (pH 8.8), 3.3 mL of 30% acrylamide, 100 µL of 10% SDS, 20 µL TEMED, and 50 µL of 10% ammonium persulfate (APS). This was then poured into the gel cassette, covered with isopropyl alcohol and left to polymerise for about 15 min.The stacking gel (also 10 mL) was made from 6.1 mL water,

2.5 mL of 0.5 M Tris-HCl (pH 6.8), 1.3 mL of 30% acrylamide, 100 µL of 10% SDS, 20 µL TEMED, and 100 µL of 10% APS. It was poured on top of the solidified separating gel and left to polymerise for 30–45 min with a comb inserted to form the loading wells.The electrophoresis tank was filled with 2× tank buffer consisting of 6.04 g Tris, 28.8 g glycine and 2 g SDS per 1 L distilled water. The stacking gel was then subjected to 100 V until the dye front was in the separating gel, and then the voltage was raised to 180 V until the dye front reached the bottom of the separating gel.

4.7.3 Coomassie Staining and Destaining

Gels were transferred after electrophoresis to a staining solution of 0.3 g Coomassie Brilliant Blue R-250 in 50 mL of methanol and 10 mL of acetic acid made up to 100 mL with water. Gels were allowed to sit on gel rocker overnight. The following day the staining solution was removed and 1 L of destaining solution (300 mL of methanol and 100 mL of acetic acid in 1 L of water) was placed on the plates. The destaining was continued for 3-4 hours on a rocker until clear bands could be seen in the clear background. The destained gels were then imaged with gel documentation system.

4.7.4 Western Blotting

Transfer and Membrane Staining

Gels were transferred onto nitrocellulose membranes by the tank transfer system for Western blot analysis (Gels 3 and 4). The towbin buffer (pH 8.3) was prepared by dissolving 3.03 g Tris base, 14.4 g glycine, 100 mL methanol and 0.2 g SDS in 1 L of water. The transfer stack was prepared in the cassette holder with a foam pad, a sheet of cellulose membrane, the gel and nitrocellulose membrane, another cellulose sheet and another foam pad with no air bubbles between any of the layers. The gel side of the cassette was oriented towards the cathode (–) and the membrane side oriented towards the anode (+) when placed in the transfer tank. Transfer was carried out at 100 V at 18–20 W for 30–40 min in cold room (4 °C) using ice.The nitrocellulose membrane was stained for a short period with Ponceau solution (25 mg Ponceau S and 1.25 mL glacial acetic acid in 25 mL water) to confirm the successful transfer of proteins and then washed with water.

Blocking and Antibody Incubation

Membrane was blocked for 1 h at room temperature in blocking solution (1× wash buffer, adjusted to pH; containing 0.1% Tween-20 and 5% skim milk). Primary antibodies (anti-8x His-Tag and anti-eGFP) were diluted in the same buffer; however, using only 1% skim milk (20 µL of each primary antibody per 10 mL). This primary antibody solution was then poured onto the membrane and allowed to incubate overnight at 15-25 °C.The membrane was then washed three times for 15 min at room temperature with the wash buffer containing freshly added 0.1% Tween-20 and incubated with secondary antibody (20 µL/10 mL of 1% skim milk buffer) for 2 h at room temperature. After 3 further washings (15 min each), the membrane was

developed by applying 1× DAB with hydrogen peroxide which was diluted from 10× stock immediately before use, and 10 mL of this working solution was poured onto the membrane. The color development was allowed to proceed for 10 – 15 min and then the reaction was stopped and the membrane was pictured.

4.8 Data Analysis and Protein Storage

4.8.1 Analysis of SDS-PAGE and Western Blot Results

The molecular weights of protein bands detected after imaging were estimated by comparing them to the protein ladder which was run in a separate lane on each gel (imaging the Coomassie-stained gels, Section 4.7.3; and the Western blot membranes, Section 4.7.4). The expected molecular weight of AtHKT1;1 was determined by its amino acid sequence. Bands that eluted at or close to the predicted size of AtHKT1;1 were deemed to be the expressed and purified protein AtHKT1;1. The presence of a specific signal at the expected molecular weight in Western blots was indicative of the identity of the 8x His tagged protein.

4.8.2 Storage of Purified Protein

The purified AtHKT1;1 protein was kept at –80 ℃ until further analysis.

CHAPTER 5
Results and discussion

5.1 Expression and Purification of AtHKT1;1 Using Method 1(Ultracentrifugation Method)

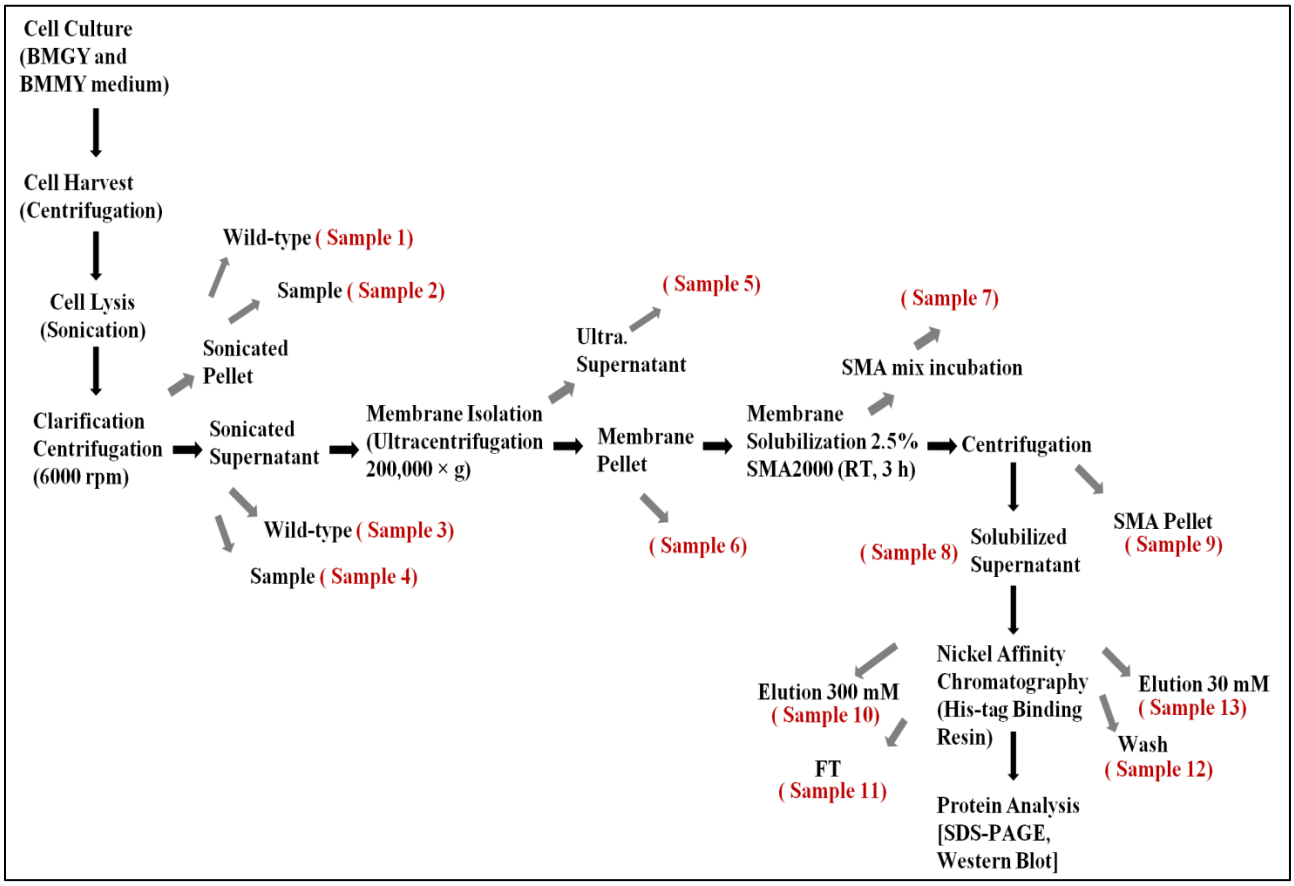

Fig.8: Flowchart depicting the sample collection strategy for Method 1

5.1.2SDS-PAGE Analysis

No major contaminants were observed in the lanes of the Coomassie-stained SDS-PAGE for the WT supernatant and wash (lanes 1–2). The soluble and membrane proteins showed complex banding patterns in lanes 3–5 (sample supernatant, ultracentrifuge supernatant, and SMA-solubilized supernatant). Weak bands were found in lanes 6-7 (flow-through and elution) indicating low protein recovery. Lanes 9-10 (SMA fractions) had dark backgrounds which may represent bands of SMA copolymer or insoluble material, and lane 11 (untransformed pellet) had a few bands. AtHKT1;1 was too scarce to be separated from the yeast and was not detected in Coomassie stained gels. We will continue with Western blotting using anti-His and anti-eGFP antibodies to confirm the bands present at ~63 kDa (possible monomer band), and another ~135 kDa band (possible dimeric form).

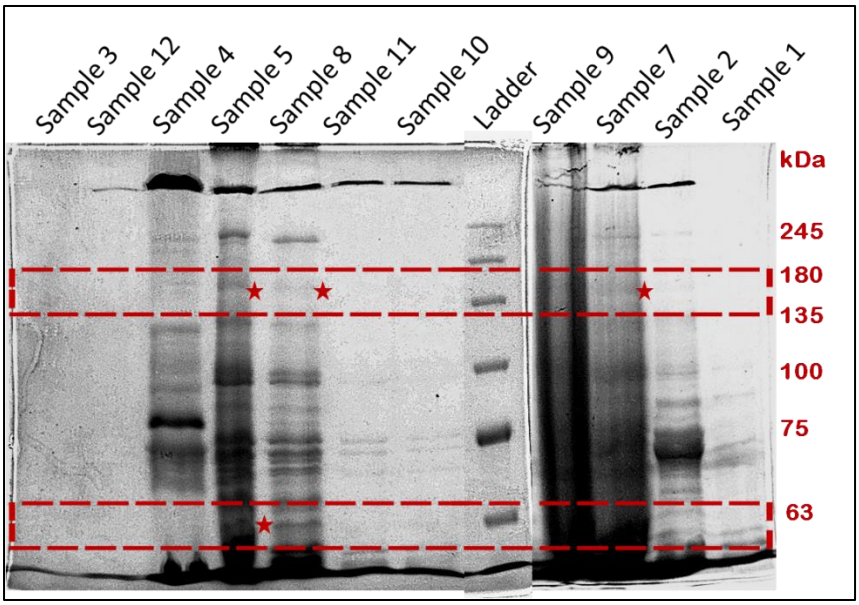

Fig.9: SDS-PAGE gel in gel documenter for the direct Sonication method (method 1). The sample order on the gel can be referred to the Fig.8

5.1.2 Western Blot Confirmation.

Western-blot using Anti-eGFP

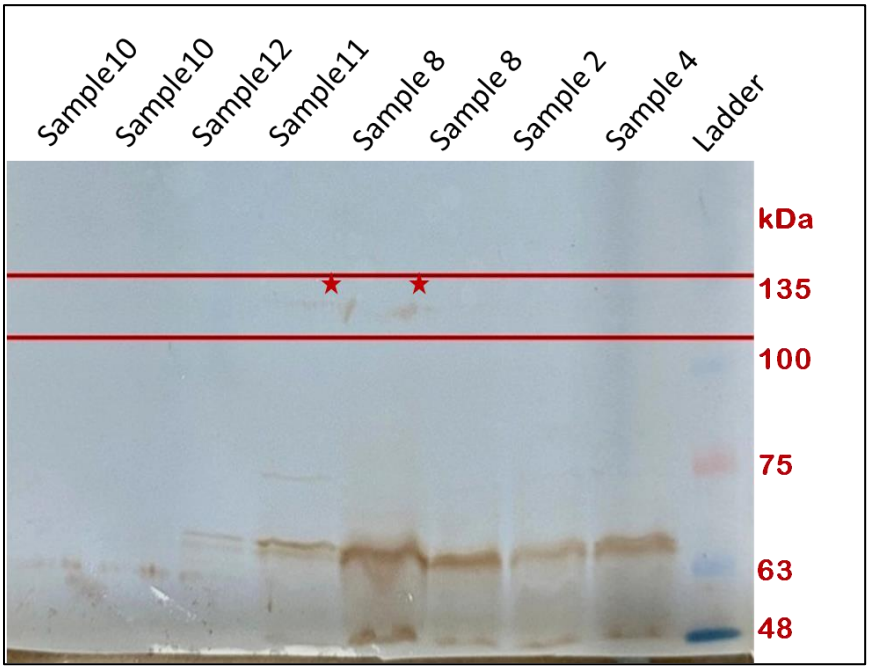

Fig.10: (10% SDS gel) Western-blot using anti-eGFP. The sample orders on the membrane canrefer to Fig.8

Western blot (Figure 10) using anti-eGFP antibody showed that the gene encoding *athkt1;1*-eGFP was expressed. Low recoveries of faint bands at ~63 kDa were observed in elution fractions (lanes 1–2, 300 mM imidazole). A very light band was also seen in washing (lane 3), and the flow-through (lane 4) was lighter indicating a degree of column binding loss. However, SMA supernatants (lanes 5–6, lane 5 being the most prominent) and Sonicated samples (lanes 7–8) exhibited strong bands just above 63 kDa, indicative of efficient solubilization and high target enrichment in these fractions.

Western-blot using anti-His

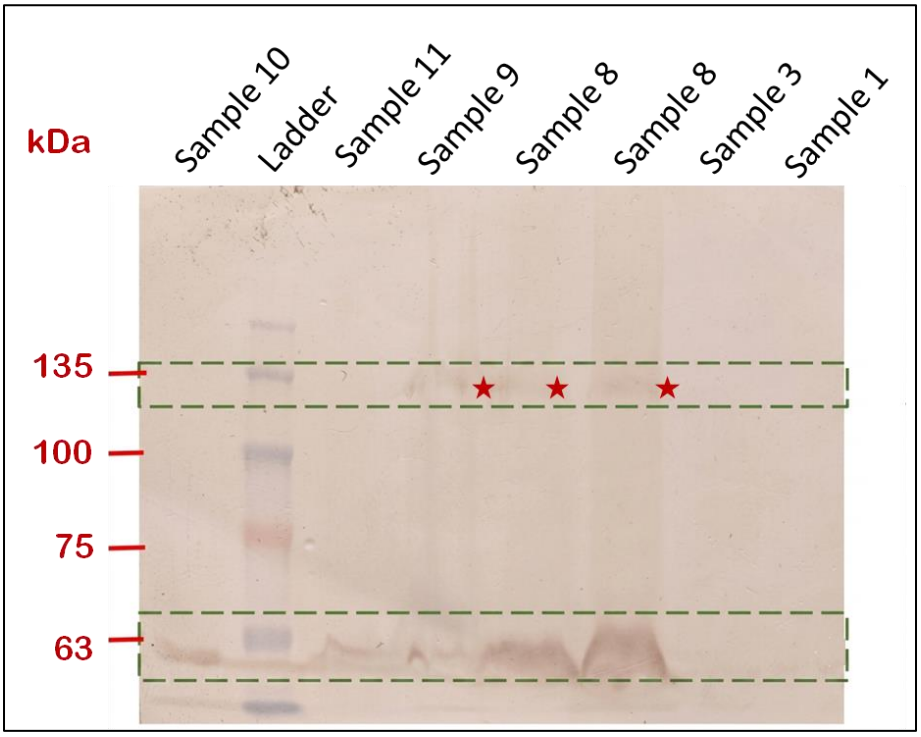

Fig.11: (10% SDS gel) Western-blot using anti-His.The sample order on the membrane can be referred from Fig.8

The expression of AtHKT1;1-eGFP-8xHis was confirmed by western blot using anti-His antibody (Figure 11). In the elution fraction (lane 1), a distinct band of ~63 kDa was detected, which is the same as the monomeric size (lane 2: marker). We observed an occurrence of partial loss during the binding of the column, which was apparent from a faint band in the flow-through lane (lane 3) at ~63 kDa. The pellet (lane 4) and supernatants (lane 5–6) of SMA fractions showed ~63 kDa bands and ~135 kDa bands (probable dimer) with much higher intensity in the supernatants, indicating efficient solubilization. Avoiding the possibility of endogenous yeast cross reactivity, no bands were seen in wild type controls (lanes 7–8), confirming antibody specificity.

5.2 Expression and Purification of AtHKT1;1 Using the Zymolyase-Spheroplast Method

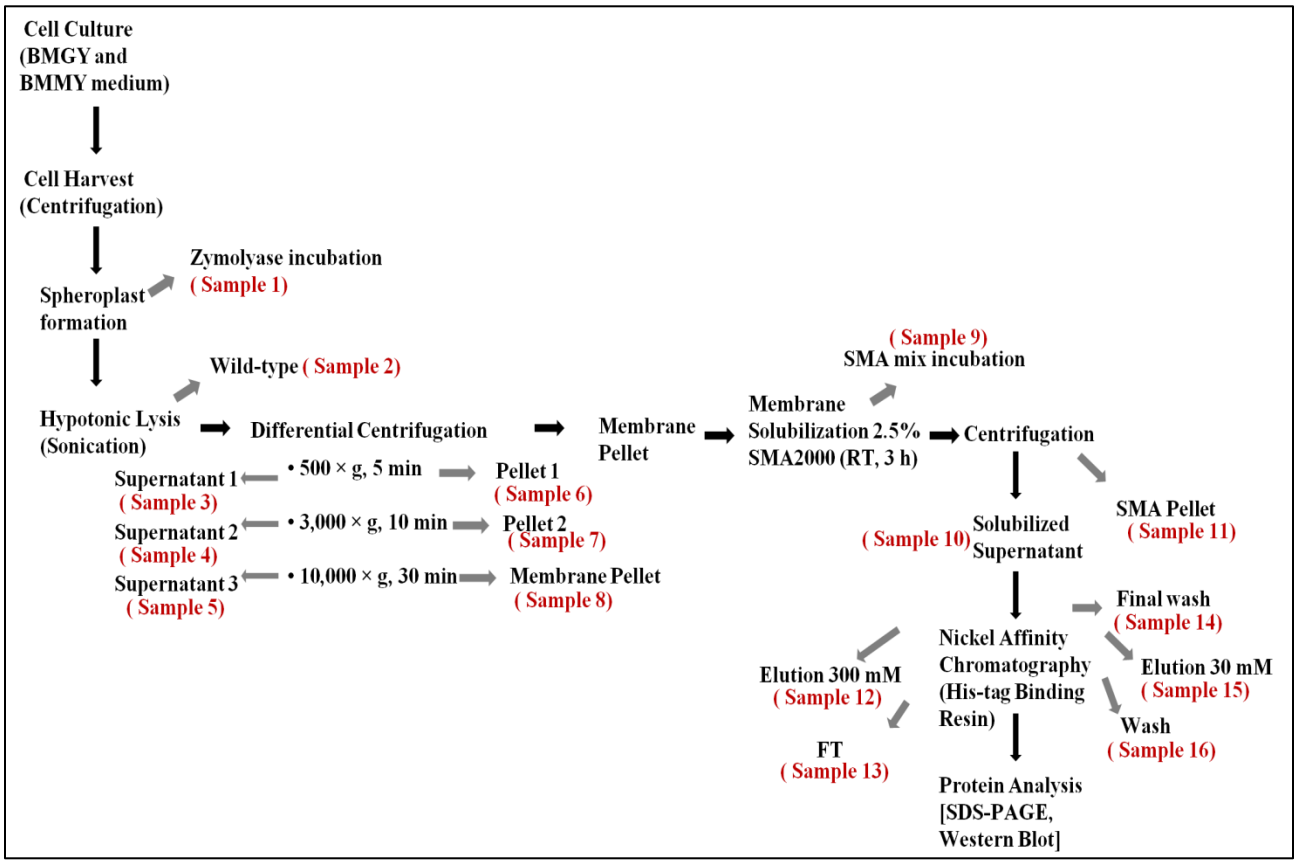

Fig.12: Flowchart depicting the sample collection strategy for Method 2

5.2.1 SDS-PAGE Analysis and Western Blot Confirmation after Solubilization withSMA2000

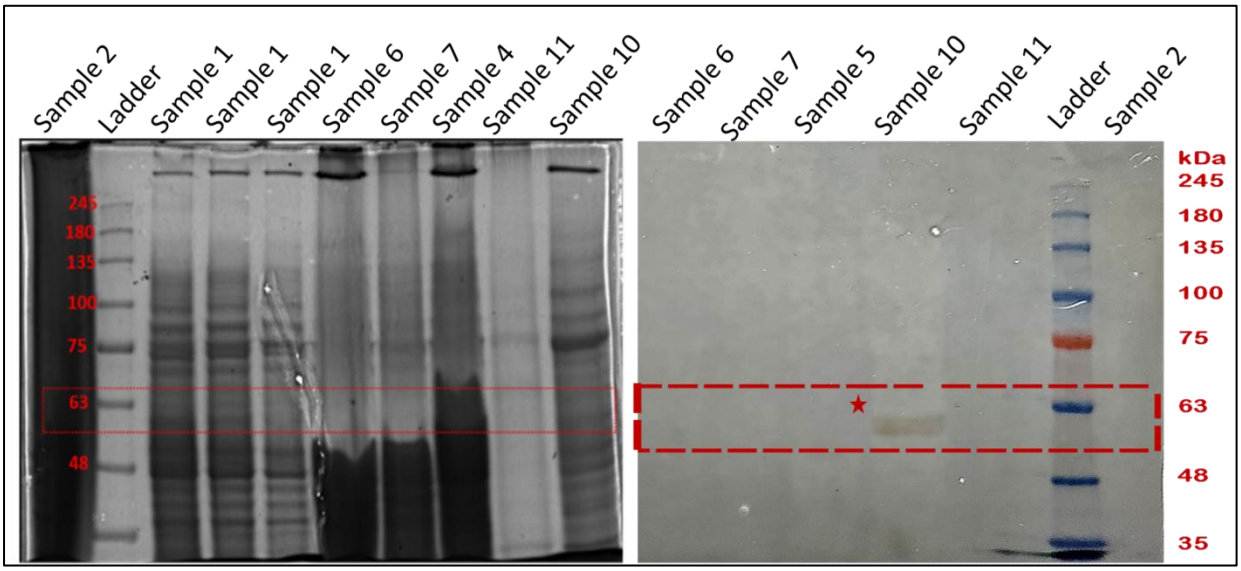

Fig.13: 5.2.1 SDS-PAGE Analysis and Western Blot Confirmation after Solubilization with SMA2000. 3, the sample order on the membrane can be referred from Fig.12

Western blotting (anti-His) was used to confirm AtHKT1;1 presence and solubilization prior to Ni^{2+}-NTA purification. The supernatant from SMA (lane 4) revealed a strong band at ~63 kDa, which showed efficient extraction into the nanodiscs. Differential centrifugation pellets (lanes 1–3), SMA pellet (lane 5) and wild type control (lane 7) did not show any signal, which indicates no protein loss, no complete solubilization and antibody specificity. An SDS-PAGE gel of the samples was also run alongside. These results confirm that the protein is suitable for the next step in the purification, which is Nickel affinity purification.

5.2.2 SDS-PAGE Analysis after Purification using Nickel Affinity Chromatography

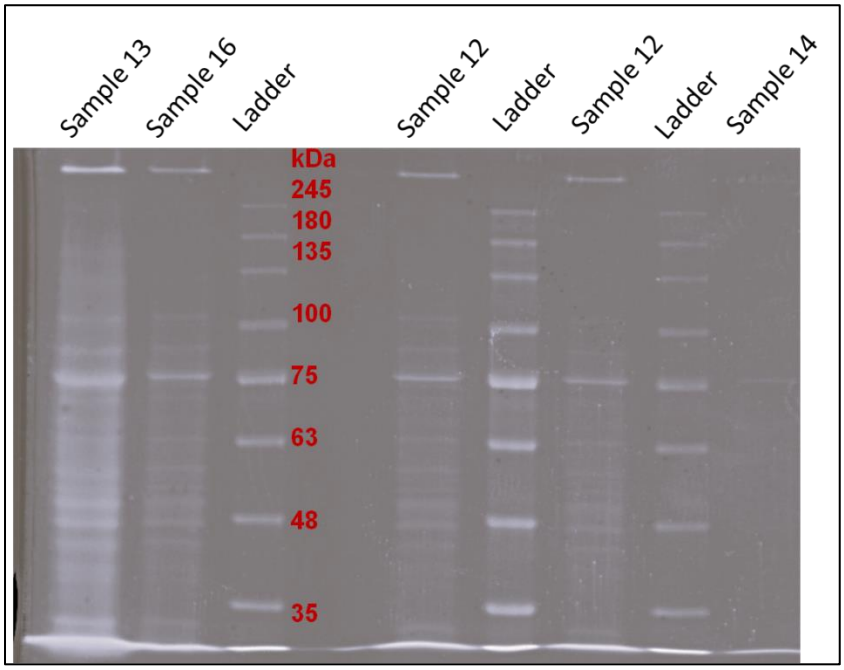

Fig.14:SDS-PAGE gel after Purification using Nickel Affinity Chromatography in gel documenter. The sample order on the gel can be refer to Fig.12

The bands in Ni^{2+}-NTA flow-through (lane 1) were detected by SDS-PAGE using Coomassie staining (Figure 14). The wash (lane 2) showed lighter bands generally, but a ~75 kDa band (which is probably AOX1) remained. The light banding pattern was similar in elution fractions (lanes 5 and 7) with the ~75 kDa contaminant, and essentially there was no contaminant in the final wash (lane 9) (lanes 3 and 6: markers, lanes 4 and 10: empty). Notably, this ~75 kDa band is not the target AtHKT1;1 (~63 kDa), and thus, further western blot analysis is required to identify the recombinant protein within these fractions.

5.2.3 Western Blot Confirmation after Purification using Nickel AffinityChromatography

After nickel affinity chromatography, fractions collected were analyzed by Western blot using an anti-His antibody to specifically detect the recombinant AtHKT1;1 (Figure 15).

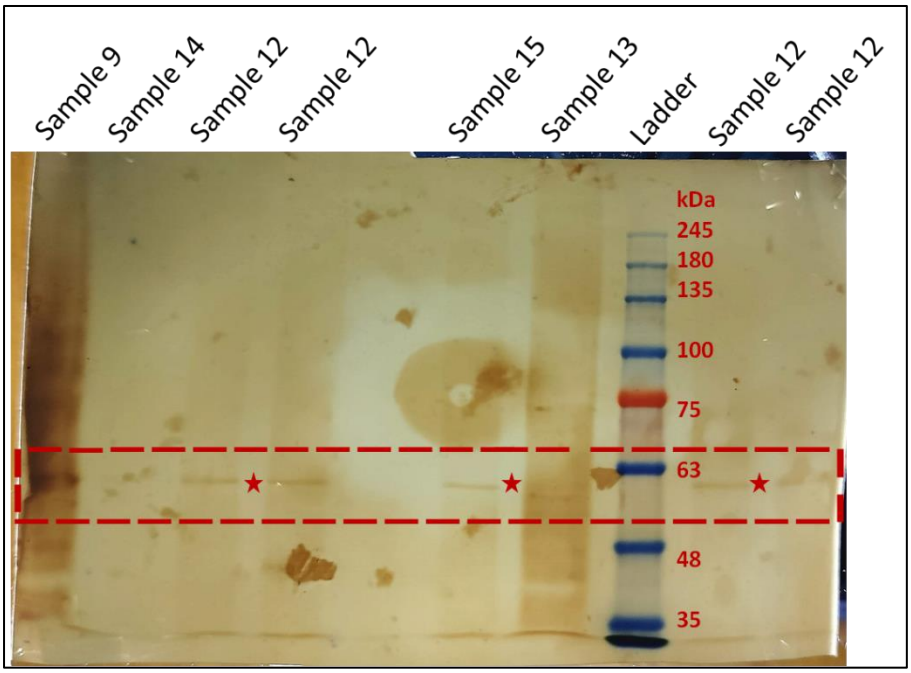

Fig.15: Western-blot using anti-His after Purification using Nickel Affinity Chromatography. The sample order on the membrane can be refer to Fig.12

Western blotting of the Ni-NTA fractions revealed the soluble protein to be a dark smear with signs of protein degradation, probably caused by the age and/or instability of the sample. The final wash (lane 2) was clear showing that unbound proteins were removed completely. A protein eluted from the column too early (lane 6, 30 mM imidazole) and a band at ~63 kDa in the flow through (lane 7) suggested that some protein did not bind the column. Importantly, the elution fractions (lanes 3, 4, 9, 10) gave a consistently good yield of the band corresponding to the ~63 kDaprobable monomeric AtHKT1;1, demonstrating successful purification (lane 5: empty).

5.3 Comparative Summary and Interpretation of Results

After following these two separate protocols (Method 1 and Method 2), it can be seen that our protein AtHKT1;1 was successfully purified by both methods, as confirmed by Western blot analysis. In both cases, a band around 63 kDa level was seen, which corresponds to the probable monomeric form of the protein. The ultracentrifugation method also showed an extra, less prominent band around 135 kDa which is probably the dimer band. The molecular weight of full-length AtHKT1;1 with the tags of 8x His and eGFP is calculated to be about 84.26 kDa. The observed band is therefore the monomeric band which is smaller than expected at ~63 kDa. There are a number of possible reasons for this difference. The first disadvantage is that, in membrane protein expression such as, the protein may sometimes migrate on SDS-PAGE at a molecular weight lower than its actual value, caused by incomplete denaturation or atypical conformation of the protein. Second, the SDS may not have been fully able to access the protein,

which is often seen with hydrophobic membrane proteins that still retain some secondary structure after boiling in SDS. Third, it is conceivable that the protein was processed or degraded at its N-terminus or C-terminus during expression or purification; however, the His tag and eGFP tag are both attached to the C-terminus in this construct, therefore the presence of both tags detected by anti-His and anti-eGFP antibodies confirms that the C-terminus has remained intact. Whatever the case, the Western blot results clearly confirm that the band identified is the one of the 63 kDa recombinant AtHKT1;1. Future work should use mass spectrometry or size-exclusion chromatography to confirm the molecular mass and evaluate for post translational modification within the purified protein, in order to conclusively determine the identity and integrity of the purified protein.

CHAPTER 6
Conclusion

The main objective of this thesis was to over-express, isolate and purify the plant salinity tolerance membrane protein AtHKT1;1 in the heterologous yeast host, *Pichia pastoris*. Two parallel methods of membrane isolation were tested: direct Sonication followed by ultracentrifugation method, and Zymolyase mediated spheroplastSonication with differential centrifugation. In both approaches, the protein was extracted with the styrene-maleic acid copolymer SMA2000 and was purified by Ni-NTA affinity chromatography using a C-terminal 8xHis tag. Both anti-His and anti-eGFP antibody consistently detected a band at the approximate size of 63 kDa (probable monomer); in the ultracentrifugation method, a band was additionally observed at the approximate size of 135 kDa (possible dimer). No bands were detected in wild-type control samples, indicating specificity of detection.AtHKT1;1 with tags is expected to have a full-length molecular weight of ~84.26 kDa. The observation of the ~63 kDa band is smaller, possibly due to N-terminal processing or anomalous migration during SDS-PAGE of hydrophobic membrane proteins. Importantly, the His and eGFP tags are attached to the C-terminus, which means that the anti His and anti eGFP antibodies are detecting the C-terminus part of the protein, and thus confirming that the C-terminus is not altered, and that the protein expressed is the actual recombinant AtHKT1;1.

This thesis addressed the successful expression, solubilization, purification and immunological detection of AtHKT1;1 in *Pichia pastoris*. Both methods of isolation were successful, and the ultracentrifugation method further showed the presence of a dimeric species. The findings in this work set the stage for a reproducible framwork for purifying AtHKT1;1, and offer a building block for future directions. The purified protein (in SMA2000 nanodiscs) can be used for mass spectrometry based identification of protein and lipid interactome. The oligomeric state and the size discrepancy in molecular mass could be confirmed by size-exclusion chromatography and native mass spectrometry. The *Pichia* expression pipeline can be scaled to yield milligram amounts to aid in the determination of structure by cryo-electron microscopy. In combination, this work enables AtHKT1;1 to become a biochemically tractable target for understanding salinity tolerance mechanisms in plants, moving beyond the genetic study of this entity.In summary, this study successfully establishes AtHKT1;1 as a biochemically accessible protein, enabling future detailed investigations.

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Total Pages, Total Words	54, 15465
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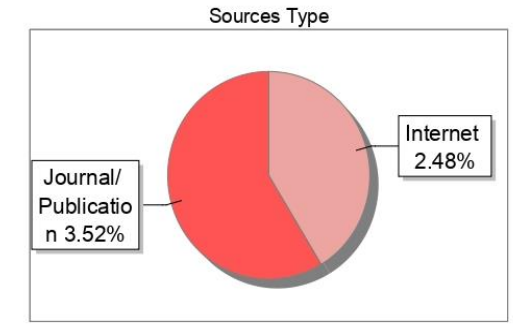

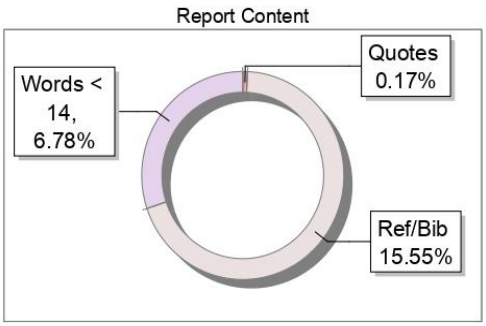

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