Molecular Cloning and Overexpression of *Arabidopsis thaliana* 14-3-3κ Protein in *Escherichia coli*

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

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

Submitted by

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CERTIFICATE

This is to certify that thesis entitled **"**Molecular Cloning and Overexpression of *Arabidopsis thaliana* 14-3-3**κ** Protein in *Escherichia coli***."** submitted by **Ms. Renu Chaudhary (Roll no. 602304009)** in the partial fulfilment of the requirements for the award of the degree of **Master of Technology 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 full to any other university or institute for the award of any other degree.

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DECLARATION

I hereby declare that the work presented in the thesis "Molecular Cloning and Overexpression of *Arabidopsis thaliana* 14-3-3**κ** Protein in *Escherichia coli*." in the partial fulfilment of the requirement of the award of the degree of Masters of Technology in Biotechnology at Thapar Institute of Engineering and Technology (TIET), is an original and genuine work completed by me between August 2024 and July 2025. 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.

Place – Patiala Renu Chaudhary

Date – 31st July 202

ACKNOWLEDGEMENT

I consider myself incredibly fortunate to have received support from various sources, including the department, my family, friends, and colleagues, both locally and internationally. Without their support, it would not have been possible for me to complete my thesis successfully.

I would like to express my sincere gratitude to my thesis guide, Dr. Debajyoti Dutta, for his invaluable guidance and support throughout the duration of this thesis. His expertise, knowledge, and unwavering dedication have been instrumental in shaping and refining this research work. I am privileged to have worked under his mentorship, and I am deeply thankful for his contributions to my academic and personal growth.

I would like to extend my sincere appreciation to Dr. MS Reddy, the Head of the Department, for providing me with the opportunity to pursue this thesis.

I owe my deepest gratitude towards Ms. Tanya Bhatnagar for her constant support. I am truly grateful for her willingness to dedicate time and effort to address my queries, provide feedback, and offer valuable suggestions. Her presence and responsiveness have been instrumental in ensuring the smooth progress of my thesis work.

I would like to thank, Mr. Harmeet Singh, for their positive support during my experiments and for helping me adjust to the lab environment. Their positivity and camaraderie have been greatly appreciated. I would like to acknowledge and thank all those who assisted me during this memorable year. Thank you all for your constant encouragement.

Date – 31st July 2025

Renu Chaudhary

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List of abbreviations

Abbreviation	Full Form
14-3-3	A conserved regulatory protein family in eukaryotes
ABA	Abscisic Acid
AHA2	*Arabidopsis thaliana* H^+-ATPase 2
ATPase	Adenosine Triphosphatase
BL21	Bacterial strain used for protein expression
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium Chloride
CBL	Calcineurin B-Like proteins
CDPK	Calcium-Dependent Protein Kinase
CIPK	CBL-Interacting Protein Kinase
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
ESPript	Easy Sequencing in PostScript
IPTG	Isopropyl β-D-1-thiogalactopyranoside

LB	Luria-Bertani (medium)
MAFFT	Multiple Alignment using Fast Fourier Transform
NaCl	Sodium Chloride
OD600	Optical Density at 600 nm
PBS	Phosphate Buffered Saline
PDB	Protein Data Bank
PCR	Polymerase Chain Reaction
RNAse	Ribonuclease
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SOS	Salt Overly Sensitive (Pathway)
TE buffer	Tris-EDTA buffer
TF	Trigger Factor
UV	Ultraviolet

List of symbols

Symbol	Description
Ca^{2+}	Calcium ion
Na^{+}	Sodium ion
K^{+}	Potassium ion
Cl^{-}	Chloride ion
µL	Microlitre
mL	Millilitre
rpm	Revolutions per minute
°C	Degree Celsius
µg	Microgram
kDa	Kilodalton
OD_{600}	Optical density at 600 nm
M	Molar concentration
µM	Micromolar concentration
% (w/v)	Percent weight/volume
~	Approximately

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Abstract

The 14-3-3 protein family plays a crucial role in regulating multiple physiological pathways in plants, particularly those involved in responses to both biotic and abiotic stresses. In *Arabidopsis*, 14-3-3 proteins interact with key partners to modulate signalling pathways, particularly those involved in stress tolerance. Besides, Ca^{2+} signalling in plants regulates plant transporters' activities in stress tolerance. However, Ca^{2+} influence on 14-3-3 function, stability, and binding affinity remains unclear. To explore this, we performed bioinformatic analyses of one of the *Arabidopsis thaliana* 14-3-3 variants, 14-3-3**κ**, which is known to bind plant plasma membrane SOS1 C-terminal residues. Since SOS1 is also indirectly regulated by cytosolic Ca^{2+}, we aimed to investigate whether Ca^{2+} influences the structure, function, or binding affinity of 14-3-3**κ**. *Arabidopsis thaliana* 14-3-3 sequences and structures are studied and compared with the known peptide-bound 14-3-3 structures to explore this. The cloning was accompanied by the overexpression of 14-3-3**κ** in *E. coli* following affinity purification. Future biochemical characterisation will focus on understanding the interaction between calcium and 14-3-3. This study may provide insights into the broader role of 14-3-3 and its interaction with Ca^{2+}, which can be used in developing stress-resilient crops.

1. Introduction

1.1 Overview of plant stress responses

Biotic and Abiotic factors are necessary for plant growth, development, and survival. Biotic factors involve interactions with living organisms, such as symbiotic microbes, pollinators, herbivores, pathogens, and competing plants. Abiotic factors refer to non-living environmental components, including temperature, water availability, humidity, mineral nutrients, radiation, wind, and carbon dioxide (CO_2). These factors support plant physiological processes, including photosynthesis, reproduction, respiration, and nutrient uptake.

However, when the optimal level of these biotic and abiotic factors is disrupted, it causes stress to plants and negatively impacts biochemical processes. Biotic stress arises from living organisms like pathogenic bacteria, viruses, fungi, insects, or even nearby plants that compete for essential resources, ultimately affecting plant health and growth.

Abiotic stress occurs when environmental conditions, like high salinity, flooding, droughts, extreme temperatures (cold, heat, and freezing), nutrient deficiency, or excessive radiation, exceed the plant's tolerance range. Both types of stress can affect normal metabolic processes, reduce photosynthesis, water and nutrient uptake, and even reduce crop yield and plant health (Bray et al., 2000; Schulze et al., 2002).

Plants have developed complex signalling networks that allow them to respond quickly to environmental challenges, which helps them adapt and survive (Zhu et al., 2016). These responses involve changes in gene expression, protein activity, ion movement, and metabolism, allowing plants to activate defence mechanisms and maintain cellular balance (Chinnusamy et al., 2004). A key group of regulators in these stress response pathways is the 14-3-3 proteins. These proteins are crucial because they interact with various target proteins to regulate their activity, stability, and localisation (Roberts et al., 2003). Highly conserved across different

species, 14-3-3 proteins participate in a wide range of physiological functions such as stress signaling, regulation of ion movement, and hormone pathways, thereby playing a vital role in helping plants adapt to stressful conditions (Denison et al., 2011).

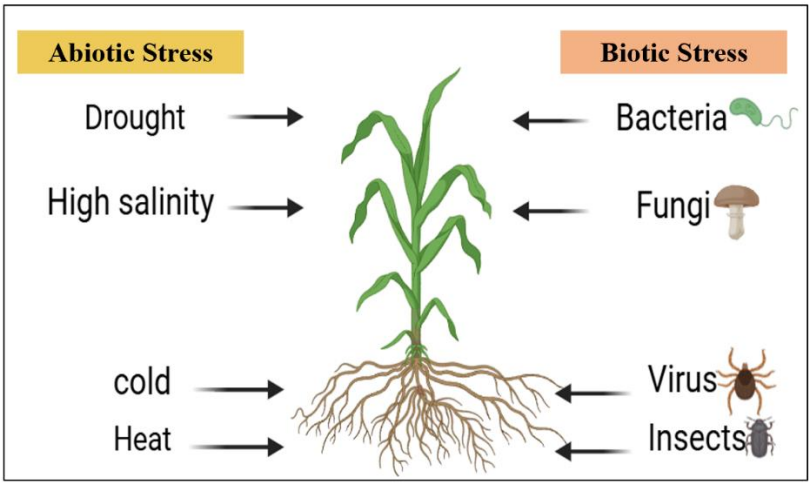

Figure 1. 14-3-3 regulates abiotic and biotic stresses.

The 14-3-3 proteins are a group of highly conserved and homologous proteins found throughout the eukaryotic kingdom. These small, water-soluble proteins typically have a molecular weight close to 30 kDa and are predominantly acidic, with an isoelectric point around pH 4.6 (Mhawech, 2005; Yaffe et al., 1997). Originally discovered in mammalian brain tissue, their designation, "14-3-3," stems from their fractional placement during early analytical procedures such as starch-gel electrophoresis and DEAE-cellulose chromatography (Aitken, 2006). In plant cells, these proteins are broadly distributed across multiple organelles, including the cytosol, nucleus, mitochondria, and chloroplasts (Bihn et al., 1997; DeLille et al., 2001; Yaffe et al., 1997). Structurally, 14-3-3 proteins are characterized by the presence of nine conserved α-helices, while their N- and C-terminal regions exhibit considerable variability. This terminal variation is believed to contribute to functional specificity and adaptability, whereas the conserved helical core supports shared roles across isoforms (Aitken, 2006; Camoni et al., 2018). Functionally, these proteins operate as dimers, comprising either

homodimers or heterodimers of different isoforms. The availability of multiple isoforms facilitates diverse dimer combinations, allowing them to regulate a variety of cellular processes. These dimers form a distinctive cup-like structure with internal grooves capable of binding phosphorylated target proteins. Each monomer within the dimer can engage a separate binding partner, enabling the dimer to interact with two phosphorylated proteins simultaneously. This unique structural configuration underpins their capacity to modulate complex signaling pathways through multiple protein–protein interactions.

Recent studies have found that the ways plants respond to biotic and abiotic stress are often connected. For example, if a plant suffers from drought, it may become more likely to get infected by germs. Similarly, it may struggle more with heat or salty soil if infected. 14-3-3 proteins help connect these different stress responses. Since they can work with many other proteins involved in both types of stress, they help the plant deal with multiple problems at once and improve its chances of survival.

Calcium is a vital secondary messenger in plant stress responses, translating external stress signals into cellular actions. For example, calcium regulates the activity of SOS3 in salt stress tolerance (Liu & Zhu et al., 1998). It activates CBL-CIPK complexes, where CBLs (Calcineurin B-like proteins) sense calcium changes and interact with CIPKs (CBL-Interacting Protein Kinases) to regulate ion transport under salt and drought stress (Batistic & Kudla, et al., 2009). These interactions show how calcium modulates protein function during stress. However, the direct role of calcium in influencing the structure, stability, or binding ability of 14-3-3 proteins remains largely unexplored.

2 Review of literature

The 14-3-3 protein family is structurally well-conserved across a wide range of organisms, highlighting its evolutionary significance. Despite this conservation, the number of 14-3-3 genes varies among species. Simpler organisms like yeast contain only two genes encoding 14-3-3 proteins, whereas more complex organisms such as humans have up to 15 (Wet et al., 2005; Met et al., 2001). In mammals, including humans, seven distinct isoforms of 14-3-3 proteins have been identified namely β, γ, ε, η, σ, τ/θ, and ζ. All of these isoforms, with the exception of sigma (σ), are capable of forming both homodimers and heterodimers. This dimerization ability is essential for their functional versatility and regulatory roles within the cell (Aitken et al., 2002; Wilker & Yaffe, 2004).

In the model plant *Arabidopsis thaliana*, the 14-3-3 protein family was initially referred to as G-box factor 14-3-3 (GF14), with the corresponding genes named as general regulatory factor (GRF) genes such as GRF1, GRF2, GRF3, and so forth. Eventually, these proteins were renamed using Greek letters, including χ (chi), ω (omega), and ψ (psi), among others (Chevalier et al., 2009). However, a universally accepted naming system for 14-3-3 proteins across plant species has yet to be established. Consequently, the nomenclature varies depending on the species. In *Arabidopsis*, for instance, there are 15 genes encoding 14-3-3 proteins, with 13 of them designated GRF1 through GRF13 (Cao et al., 2007; Chevalier et al., 2009; Rosenquist et al., 2001). In the case of tomato (*Solanum lycopersicum*), twelve genes have been identified and are labeled TFT1 to TFT12 (Roberts, 2003; Xu et al., 2013; Xu & Shi et al., 2006). Rice (*Oryza sativa*) possesses eight 14-3-3 genes, referred to as GF14a through GF14h (Chen et al., 2006; Chevalier et al., 2009; Yao et al., 2007). Meanwhile, the tobacco family contains 17 members of this protein family, which are systematically named from 14-3-3 a-1 to 14-3-3 i-2 (Roberts, 2003; Xu et al., 2013; Xu & Shi et al., 2006).

In *Arabidopsis thaliana*, the 14-3-3 protein family is categorized into two main subgroups based on variations in their gene structure and amino acid composition. The first subgroup, known as the ε-group, typically contains genes characterized by a more complex structure, with six to seven exons and four to six introns. In contrast, the non-ε group consists of genes that are structurally simpler, generally comprising four exons and three introns (Sehnke et al., 2002).

The existing classification and variable nomenclature reflect the diversity and complexity of the 14-3-3 protein family in plants.

Research has demonstrated that 14-3-3 proteins have a high affinity for binding to target proteins at phosphorylated serine or threonine residues, a mechanism that allows them to influence numerous cellular functions. In *Arabidopsis thaliana*, for example, these proteins interact with the plasma membrane H^+-ATPase (AHA2) by recognizing a phosphorylated threonine residue at the enzyme's C-terminal end, which results in the activation of the enzyme (Fuglsang et al., 1999). They also associate with the phosphorylated form of the SOS2 kinase, playing a vital role in plant responses to salt stress (Zhou et al., 2014). Another well-studied interaction involves nitrate reductase (NR), where 14-3-3 binding dependent on phosphorylation suppresses the enzyme's function, thereby regulating nitrogen metabolism in plants (Moorhead et al., 1996). These interactions collectively emphasize the importance of 14-3-3 proteins in coordinating stress signaling pathways and metabolic regulation in plants.

These interactions suggest that phosphorylation is a key mechanism through which proteins interact with each other (Aj et al., 1996). Subsequent studies proved that 14-3-3 proteins can attached to unphosphorylated motifs, expanding their range of interaction partners beyond classical phospho-dependent binding. For example, in tobacco, 14-3-3 proteins interact with the basic leucine zipper-type (bzip), transcription factor RSG (Repression of Shoot Growth) in

a phosphorylation-independent manner, influencing its nuclear localisation and thereby regulating genes involved in shoot development (Igarashi et al., 2001). Similarly, in mammals, 14-3-3 proteins bind directly to the pro-apoptotic protein BAX (Bcl-2-associated X protein), retaining it in the cytoplasm and preventing it from triggering apoptosis by disrupting mitochondrial membranes (Nomura et al., 2003). These findings suggest that 14-3-3 proteins possess versatile binding capabilities, allowing them to participate in diverse cellular and physiological pathways across kingdoms. Due to these interactions, 14-3-3 proteins are involved in critical cellular processes, such as controlling the cell cycle, regulating apoptosis (cell death), managing metabolism, and regulating gene expression. For instance, 14-3-3 proteins regulate the cell cycle by binding to and sequestering cell cycle regulators like CDC25 phosphatases, thereby preventing premature mitotic entry. In apoptosis, they bind to BAX in mammals, keeping it in the cytoplasm and blocking its pro-apoptotic function. In metabolism, 14-3-3 proteins interact with phosphorylated nitrate reductase (NR) in plants to inhibit its activity, thereby modulating nitrogen metabolism. For gene expression, they bind to transcription factors like RSG in tobacco, retaining them in the cytoplasm and preventing them from activating growth-related genes in the nucleus.

Certain polycations, including magnesium ions (Mg^{2+}) and spermine, have been shown to enhance the binding affinity between 14-3-3 proteins and their phosphorylated targets, such as nitrate reductase (pNR) (Shen & Huber, 2006). Magnesium plays a key role in promoting the interaction between 14-3-3 proteins and plasma membrane H^{+}-ATPase, which is essential for maintaining cellular ion homeostasis. Spermine, a naturally occurring polyamine, has an even stronger influence in reinforcing these protein-protein interactions (Garufi et al., 2007). Functionally, 14-3-3 proteins act as adaptor molecules or scaffolds, facilitating interactions between signaling proteins (Bridges et al., 2005). Proteomics-based studies have identified a wide range of 14-3-3 binding partners, including proteins involved in the transport of essential

elements such as iron, potassium (K^+), calcium (Ca^{2+}), and chloride (Cl^-), as well as components of ethylene and brassinosteroid signaling pathways. Additionally, they regulate transcription factors like the WRKY family (Chang et al., 2009).

Beyond mediating interactions, 14-3-3 proteins influence multiple properties of their targets, including enzymatic activity, structural conformation, cellular localization, and stability (Chevalier et al., 2009; Obsil & Obsilova, 2011). For instance, Chevalier and colleagues reported their association with plasma membrane H^+-ATPases and phototropins in guard cells, affecting both enzyme activation and intracellular distribution. Structural studies summarized by Obsil & Obsilova further reveal that 14-3-3 proteins contribute to the stabilization and regulation of enzymes like aralkylamine N-acetyltransferase (AANAT), kinases such as DAPK2 and B-RAF, and transcription factors including FOXO4. The interaction with FOXO4, a mammalian transcription factor central to stress response and apoptosis, occurs in a phosphorylation-dependent manner, whereby 14-3-3 binding retains FOXO4 in the cytoplasm, thereby preventing it from activating gene expression in the nucleus. Such examples illustrate the broad regulatory capacity of 14-3-3 proteins, which operate through mechanisms like conformational modulation, masking of interaction sites, and stabilization of protein complexes. Moreover, they also mediate intracellular trafficking, such as the relocation of REPRESSION OF SHOOT GROWTH (RSG) from the nucleus to the cytoplasm (Igarashi et al., 2001; Ishida et al., 2004).

2.2 14-3-3 proteins in stress signalling pathways

14-3-3 proteins help plants cope with stress, including biotic and abiotic stress. These proteins enable plants to sense problems and respond immediately by adjusting cellular processes. Acting as signalling systems, 14-3-3 proteins manage energy and balance ions and water when plants face abiotic stress, such as salt stress. They help regulate many proteins responsible for

these stresses, including enzymes, transporters, and gene regulators. 14-3-3 proteins assist plants in eliminating excess salt from their cells by interacting with the SOS pathway. In plants, 14-3-3 proteins connect different signalling systems by directly interacting with other proteins. This includes pathways such as calcium (Ca^{2+}), abscisic acid (ABA), and reactive oxygen species (ROS). These interactions allow for the coordination of multiple signals, enabling plants to respond effectively to changing environmental conditions.

2.3 Calcium signalling in plants

Calcium ions (Ca^{2+}) serve as vital secondary messengers in plant cells, playing a key role in the early response to various environmental challenges such as salinity, drought, cold temperatures, and pathogen invasion. One of the immediate cellular reactions to these stresses is a swift elevation in cytosolic Ca^{2+} levels. These fluctuations, often termed "calcium signatures," are intricately regulated in terms of their intensity, duration, and spatial patterning. Specific calcium-sensing proteins such as calmodulins (CaMs), calcium-dependent protein kinases (CDPKs), and calcineurin B-like proteins (CBLs) are responsible for interpreting these calcium signals (Reddy et al., 2011). Upon sensing these signals, the calcium sensors activate downstream effectors like ion channels, transporters, and transcription factors, ultimately triggering physiological or developmental processes tailored to the stress condition. Calcium signaling plays an especially critical role under abiotic stress conditions. For example, during salt stress, the Salt Overly Sensitive (SOS) pathway is activated in response to calcium influx. In this pathway, the Ca^{2+}-bound form of SOS3 interacts with and activates SOS2, a protein kinase that subsequently regulates SOS1 a plasma membrane Na^+/H^+ antiporter that functions to remove excess sodium ions from the cell, thus helping maintain ionic balance (Dodd et al., 2010). Calcium may also influence the functions of other regulatory proteins, such as 14-3-3 proteins. While the detailed mechanisms remain unclear, Ca^{2+} may directly or indirectly affect the structure or binding ability of 14-3-3 proteins through calcium-binding intermediates. For

example, the SOS (Salt Overly Sensitive) pathway uses calcium signals to activate the CBL-CIPK module that maintains ion homeostasis under salt stress. Similarly, Ca^{2+} signals regulate abscisic acid (ABA) responses in drought conditions by modulating SnRK2 kinase activity through calcium-dependent protein kinases (CDPKs). Another example is the touch or mechanical stress response, where calcium waves activate calmodulin and CAMTA transcription factors that regulate stress-responsive gene expression. Understanding how plants integrate calcium signalling with other regulatory networks, including those involving phosphorylation and protein–protein interactions, is essential.

2.4 The SOS pathway

Salinity is a major environmental stressor that adversely influences agricultural productivity by limiting plant growth and reducing crop yields. Elevated concentrations of salt in the soil disrupt normal physiological processes, leading to stunted development and lower productivity. To overcome these challenges, plants have evolved specialized strategies, one of which is the Salt Overly Sensitive (SOS) signaling pathway. This pathway plays a critical role in maintaining sodium ion homeostasis within plant cells, enabling them to better tolerate high-salinity conditions.

Three major proteins that assist plants in minimising sodium ion accumulation and surviving in adverse conditions are SOS1, SOS2, and SOS3. Various genetic experiments have been conducted to compare salt-tolerant plants with normal ones. In *Arabidopsis thaliana*, scientists first identified these three proteins (Zhu et al., 1998). The SOS pathway involves three core components: SOS3 (a calcium sensor), SOS2 (a serine/threonine kinase), and SOS1, which helps remove toxic sodium ions from the cytoplasm by acting as a Na^+/H^+ exchanger.

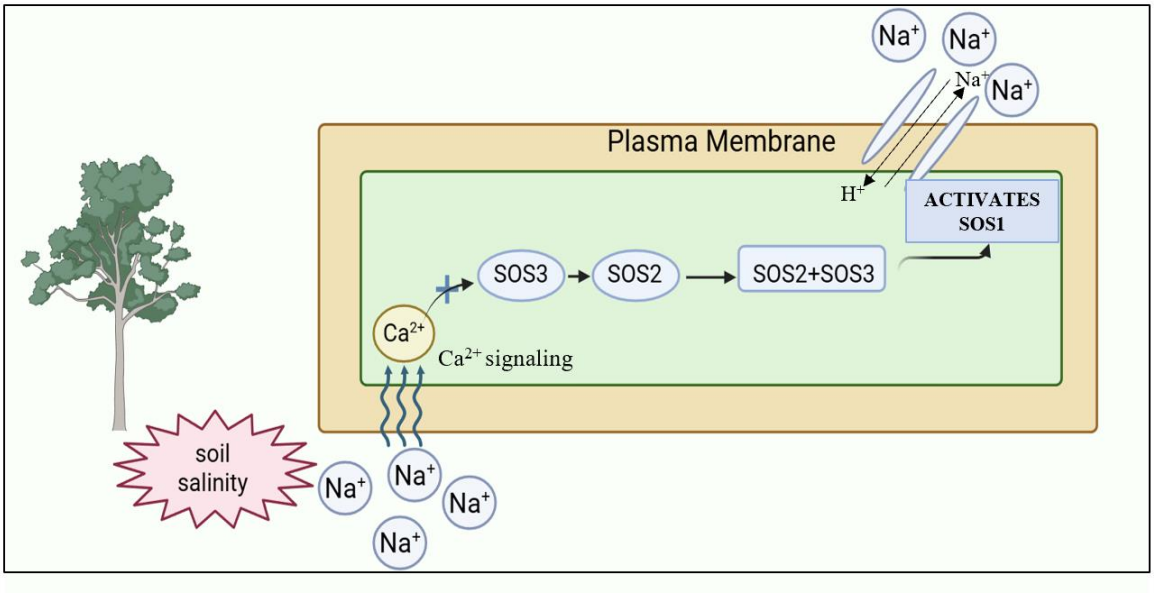

Fig 2. Salt overly sensitive (SOS) Pathway

SOS1 is primarily regulated by SOS2-SOS3. SOS3, the calcium-binding protein, binds to calcium released during stress activation and subsequently activates the SOS2 kinase (Ali et al., 2023). SOS2 and SOS3 form a complex known as SOS2-SOS3, which interacts with SOS1 to promote the release of Na^+ from cells, thereby enhancing salt tolerance (Ali et al., 2023). The SOS1 pathway can also be triggered through the action of the SCaBP8-SOS2 complex. SCaBP8, a calcium-binding protein similar to SOS3, belongs to the calcineurin B-like (CBL) protein family, which has been extensively characterized in *Arabidopsis thaliana* genome (Laun et al., 2002; Gong et al., 2004; Lin et al., 2009). CBL proteins have emerged as essential regulators of ionic balance within plant cells. For example, the cooperative interaction between CBL1 and CBL9 activates CBL-interacting protein kinase 23 (CIPK23), which in turn promotes potassium ion (K^+) homeostasis. This is achieved by increasing the activity of AKT1, a potassium channel located in the plasma membrane.

2.5 SOS1 transporter and its regulation by $Ca^{2+}$and 14-3-3

Recent advances in molecular biology and bioinformatics have identified potential 14-3-3 protein binding motifs within the C-terminal region of the SOS1 protein, indicating that 14-3-

3 proteins may play a regulatory role in SOS1 function (Yadav et al., 2020). It is proposed that phosphorylation of SOS1 by SOS2 could generate specific docking sites for 14-3-3 binding, although the precise regulatory mechanism is still not fully understood. As scaffold proteins, 14-3-3s may contribute an additional level of control by influencing SOS1 stability, activity, cellular localization, or intracellular trafficking (Paulet et al., 2012; Roberts et al., 2003). Furthermore, fluctuations in cellular calcium levels might impact the interaction between SOS1 and 14-3-3 proteins, given the known involvement of 14-3-3s in calcium-mediated signaling pathways. This suggests a possible coordinated role of calcium signaling and 14-3-3 proteins in enhancing plant adaptation to salt stress and other adverse environmental conditions (Dension et al., 2011).

Recent studies suggest that 14-3-3 proteins might interact with SOS1, forming a complex network of signals that helps plants deal with salt and other types of stress. While scientists have made good progress in understanding these systems, the exact details of how these proteins work together are still unclear. More research using biochemical, genetic, and protein analysis methods is needed to fully understand these interactions. This knowledge could eventually help in developing crops that are better able to survive stressful conditions like high soil salinity.

***Table 1*:** Different 14-3-3 proteins studied in plants

Sl.NO.	Organism Name	Comments	References
1.	*Tobacco (Nicotiana tabacum)*	Overexpression of 14-3-3 improves drought and salt tolerance by regulating ROS detoxification and stress-responsive gene expression	Yan et al., 2004
2.	*Arabidopsis thaliana*	14-3-3 proteins activate the plant plasma membrane H+-ATPase enzyme by binding to its C-terminal region	Ottmann et al., 2007
3.	*Solanum lycopersicum*	14-3-3 interacts with aquaporins, influencing root water transport under salt stress	Prado et al., 2013
4.	*Rice (Oryza sativa)*	14-3-3 proteins act as intracellular receptors for florigen, offering new strategies to control flowering in plants	Taoka et al., 2011
5.	*Arabidopsis thaliana*	14-3-3 proteins are involved in plant defense, including their response to pathogens, their interactions with defense proteins, and how they are targeted by pathogen	Duran et al., 2015
6.	*Setaria italica (foxtail millet)*	Overexpression in *Arabidopsis* leads to earlier flowering under salt stress, suggesting a role in stress adaptation.	Liu et al., 2020
7.	*Arabidopsis thaliana*	14-3-3 protein regulate processes like cell growth, seed germination, and responses to stresses such as drought, cold, and salt, often through hormone signaling	Huang et al., 2021

8.	*Hordeum vulgare* (barley)	Hv14-3-3A interacts with drought-responsive proteins, regulating stomatal closure and enhancing drought tolerance.	Jiang et al., 2023
9.	*Arabidopsis thaliana*	Mechanistic and genetic evidence highlighting the critical regulatory role of 14-3-3 proteins at multiple stages of the brassinosteroid (BR) signaling pathway	Obergfell et al., 2023
10.	*Manihot esculenta (cassava)*	Genome-wide identification suggests a role in negative regulation of starch accumulation in storage roots.	Frontiers in Plant Science, 2023
11.	*Arabidopsis thaliana*	SOS1 protein, crucial for plant salt stress tolerance and identified 14-3-3 proteins that bind to SOS1's C-terminal tail, affecting its activity	Duscha et al., 2024

3. Aim

This thesis aims to analyse the structure and function of *Arabidopsis thaliana* 14-3-3**κ** by using computational methods and to express the protein in bacteria for purification towards it *in vitro* characterization.

4. Objectives-

- To conduct a comprehensive structural and functional analysis of *Arabidopsis thaliana* 14-3-3 proteins using computational methods.
- To perform cloning of *A. thaliana* 14-3-3**κ** gene into a bacterial expression vector
- To perform expression and solubilization of *A. thaliana* 14-3-3 **κ** gene in bacteria

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5. Materials and methods

5.1 Bioinformatic analysis

The 14-3-3 protein sequences involved in abiotic stress conditions were analyzed using various bioinformatics tools. Crystal structures of 14-3-3 isoforms were retrieved from the Protein Data Bank (PDB) with the following PDB IDs: 8QT5, 2O98, 2BR9, 2O02, 1QJB, 6BQT, 2NPM, 4ZQ0, and 2O8P. The 14-3-3**κ** isoform of *Arabidopsis thaliana* (UniProt ID: P48348) was selected as the reference sequence for structural and comparative analysis.

Multiple sequence alignment of selected 14-3-3 protein sequences was performed using MAFFT (Multiple Alignment using Fast Fourier Transform) with default parameters. The aligned sequences were further analyzed and visualized using ESPript 3.0 [https://doi.org/10.1093/nar/gkg556] to highlight conserved regions, phosphorylation sites, and 14-3-3 binding motifs.

Structural visualization, domain identification, and dimerization analysis of 14-3-3 proteins were carried out using PyMOL (version 2.5.2). Conserved amino acid residues were mapped onto the 3D structure using PyMOL for the identification of potential functional regions as shown in the fig 4.

This protocol was adapted with reference to the methodology used in the study by (de Vries et al., 2021), which conducted a comparative analysis of 14-3-3 isoforms using similar bioinformatics approaches.

5.2 Cloning and Expression of 14-3-3κ

5.2.1 Isolation of the bacterial cloning plasmid and using alkaline lysis method

A single colony of *E. coli* cells containing the pCold-TF (amp^+) vector was inoculated in 50 mL LB broth containing 50 µg/mL ampicillin and incubated overnight at 37°C with shaking at

180 rpm. The overnight culture was centrifuged at 8000 rpm for 10 minutes at 4°C. The pellet was resuspended in 400 µL of Re-suspension Buffer (50 mM Tris-HCl Ph 8.0, 10 mM EDTA, econtaining RNase A) and incubated on ice for 10 minutes.

Lysis was performed by adding 800 µL of Lysis Buffer (0.2 M NaOH, 1% SDS), followed by gentle mixing through inversion (8–10 times). The lysate was incubated on ice for 10 minutes. Neutralization was done by adding 600 µL of Neutralization Buffer (3 M potassium acetate and glacial acetic acid, pH 5.5), mixing, and incubating again on ice for 10 minutes. The mixture was centrifuged at 12,000 rpm for 10 minutes at 4°C, and the clear supernatant was transferred to a fresh tube.

An equal volume of isopropanol was added to the supernatant, mixed by inversion, and incubated at room temperature for 10 minutes. The mixture was centrifuged at 10,000 rpm for 10 minutes at 4°C. The resulting DNA pellet was washed with 70% ethanol, air-dried, and resuspended in 50 µL TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

For further purification, 350 µL distilled water and 2 µL RNase were added, and the mixture was incubated at 37 °C for 40 minutes. Equal volume of phenol:chloroform (1:1) was added, followed by centrifugation at 10,000 rpm for 10 minutes. The aqueous phase was transferred to a clean tube, and DNA was precipitated by adding 40 µL of 3 M sodium acetate and an equal volume of absolute ethanol. Samples were stored at –20 °C overnight. The next day, centrifugation was carried out at 12,000 rpm for 15 minutes. The DNA pellet was washed with 100% ethanol, air-dried, and dissolved in 50 µL TE buffer.

5.2.2 Restriction digestion and gel purification

To verify the presence and orientation of the insert, plasmid DNA was digested with ***Nde*I** and ***Pst*I** at 37 °C for 2 hours. The resulting fragments were separated on a 1% agarose gel and visualized under UV light.

The desired DNA band was excised from the gel and weighed. For every 100 mg of gel slice, 300 µL of Qiagen Buffer QG (QIAGEN Inc.) was added. The sample was incubated at 50 °C with intermittent vortexing until the gel was fully dissolved. Afterward, an equal volume of isopropanol was mixed in, and the solution was transferred to a silica membrane spin column. The column was washed using 750 µL of Buffer PE, and the purified DNA was eluted and stored at –20 °C. Both the synthetic gene and the pCold-TFexpression plasmid were subjected to digestion with the same enzymes, run on agarose gel, and the appropriate bands were extracted for ligation.

5.2.3 Transformation into *E. coli*

Transformation of *E. coli* was performed using two methods: calcium chloride-mediated transformation and electroporation.

A. Calcium chloride transformation

A fresh 100 mL *E. coli* culture was grown to mid-log phase (OD_{600} = 0.4–0.6) at 37 °C. The cells were harvested at 7000 rpm for 10 minutes at 4°C and resuspended in 10 mL of ice-cold 0.1 M $CaCl_2$. After a 15-minute incubation on ice, the cells were pelleted again and washed with 5 mL of the same chilled buffer, then finally resuspended in 1 mL of cold 0.1 M $CaCl_2$ and incubated on ice for 2 hours to render the cells competent.

Two tubes were prepared:

- 100 µL competent cells + 5–10 µL plasmid DNA
- 100 µL competent cells (control)

The tubes were kept on ice for 30 minutes, heat-shocked at 42 °C for 2 minutes, then placed on ice again for 2 minutes. After recovery in 1 mL of LB broth at 37°C for 1 hour (100 rpm),

100 µL from each culture was plated on LB agar containing ampicillin and incubated overnight at 37 °C.

B. Electroporation

The bacterial culture was centrifuged and washed three times with sterile, ice-cold water. The final cell pellet was resuspended in 100 µL of ice-cold water and divided into two tubes:

- 50 µL cells + 5 µL plasmid DNA
- 50 µL cells without DNA (control)

The mixtures were transferred into a 0.2 cm electroporation cuvette and pulsed at 1.8 kV. Immediately after the pulse, 1 mL LB broth was added, and the cultures were incubated at 37 °C for 1 hour at 1000 rpm, followed by plating on LB-ampicillin agar.

5.2.4 Colony selection and culture preparation

Following transformation, eight colonies were selected for analysis three from the calcium chloride method, three from electroporation, and two from the electroporation control. Each colony was streaked onto a fresh LB-ampicillin plate and also inoculated into 2 mL of LB broth (with ampicillin) in sterile Falcon tubes. Controls included untransformed *E. coli* BL21 and *E. coli* containing an empty p Cold-TF vector. All cultures were incubated overnight at 37 °C with shaking at 180 rpm.

5.2.5 IPTG induction for protein expression

To initiate protein expression, 5 mL from each overnight culture was inoculated into 50 mL of fresh LB broth supplemented with ampicillin (100 µg/mL). The cultures included 3 colonies transformed via electroporation (EL1, EL2, and EL3), 3 colonies transformed using the heat shock method (HS4, HS5, and HS6), along with BL21 and an empty p Cold-TF vector control. Once the cultures reached an OD_{600} between 0.4 and 0.6, protein expression was induced by

adding IPTG to a final concentration of 0.5 mM. The cultures were then incubated at 16 °C for 24 hours with continuous shaking. Following incubation, cells were harvested by centrifugation at 8000 rpm for 10 minutes at 4 °C, and the resulting cell pellets were resuspended in 1.5 mL of resuspension buffer for downstream processing.

5.2.6 Protein extraction, quantification, and analysis

Cells were lysed by sonication on ice using short pulses over a 15-minute period. The lysates were centrifuged at 8000 rpm for 10 minutes at 4 °C to separate the soluble protein fraction, which was stored at –20 °C for further analysis. Protein concentration was measured using a spectrophotometer (e.g., NanoDrop). Expression levels were assessed by resolving the samples on a 12% SDS-PAGE gel. Protein bands were visualized, and the molecular weight of the expressed 14-3-3κ protein was compared to a pre-stained protein ladder.

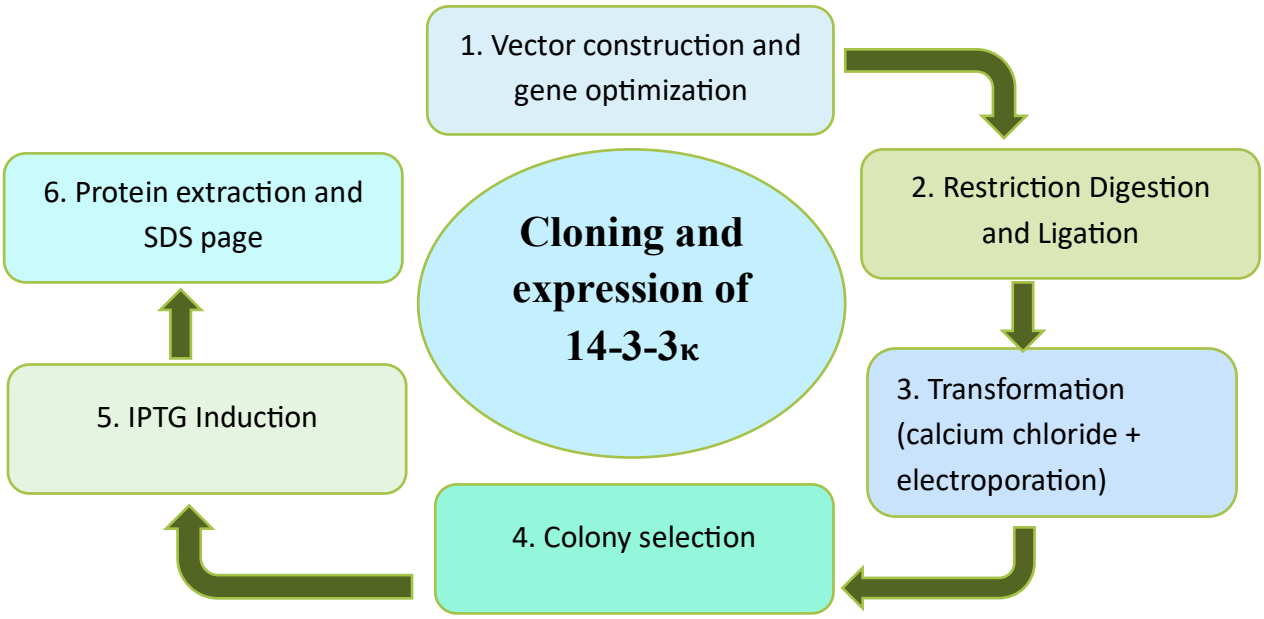


Fig.3. Workflow for recombinant cloning and overexpression of of A. *thaliana* 14-3-3κ gene in *E. coli*

6. Result and Discussions

6.1 Multiple Sequence Alignment and Secondary Structure Analysis of 14-3-3 Proteins

Multiple sequence alignment of 14-3-3 protein isoforms from various species was performed using MAFFT and visualized with ESPript. A multiple sequence alignment (MSA) was done to compare 14-3-3 proteins from different species to understand how similar they are and how their structure is conserved. The alignment includes the 14-3-3 protein from *Arabidopsis thaliana* (AR P48348), different Homo sapiens (like HS 8QT5, HS 2098, HS 2BR9), and proteins from other plants like *Nicotiana tabacum* (NT), *Giardia Lamblia* (GL), and *Cryptosporidium Parvum* (CP). In the alignment, red-colored regions show amino acids that are the same in most species, which means they are highly conserved and likely very important for the protein's function.

The figure also shows secondary structures, including α-helices (labeled α1 to α10) and one short η1 helix, which are common in 14-3-3 proteins. Some helices like α3, α5, α7, and α9 are especially well conserved and are known to help the protein bind to other molecules or form dimers (pairs). A special region called the amphipathic groove, which helps the protein interact with partner proteins, is also conserved.

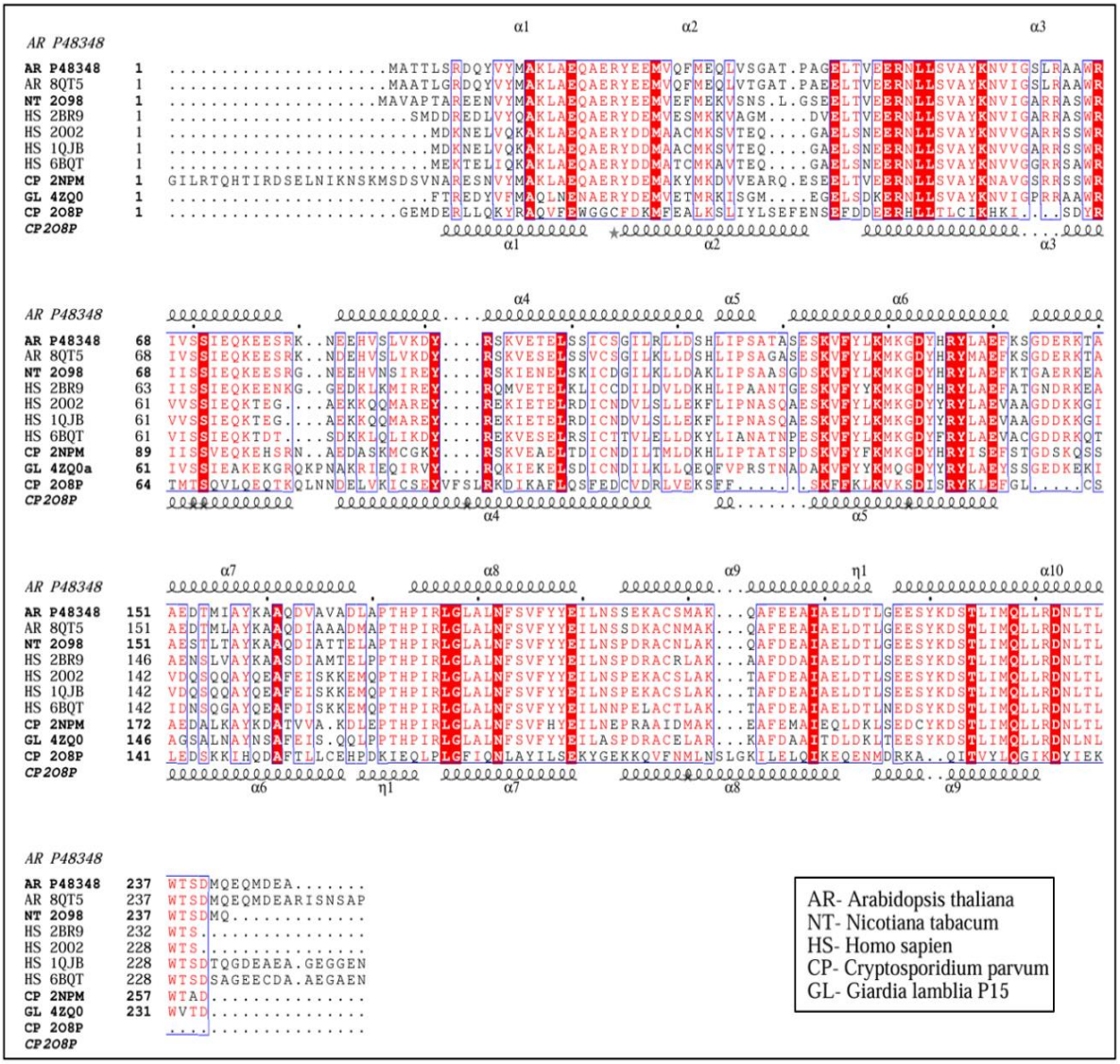


Fig 4. Multiple sequence alignment of 14-3-3 protein isoforms across various organisms highlighting conserved residues and secondary structural elements

Table 2. Key structural and functional aspects of 14-3-3 protein interactions with phosphopeptides across different isoforms and species

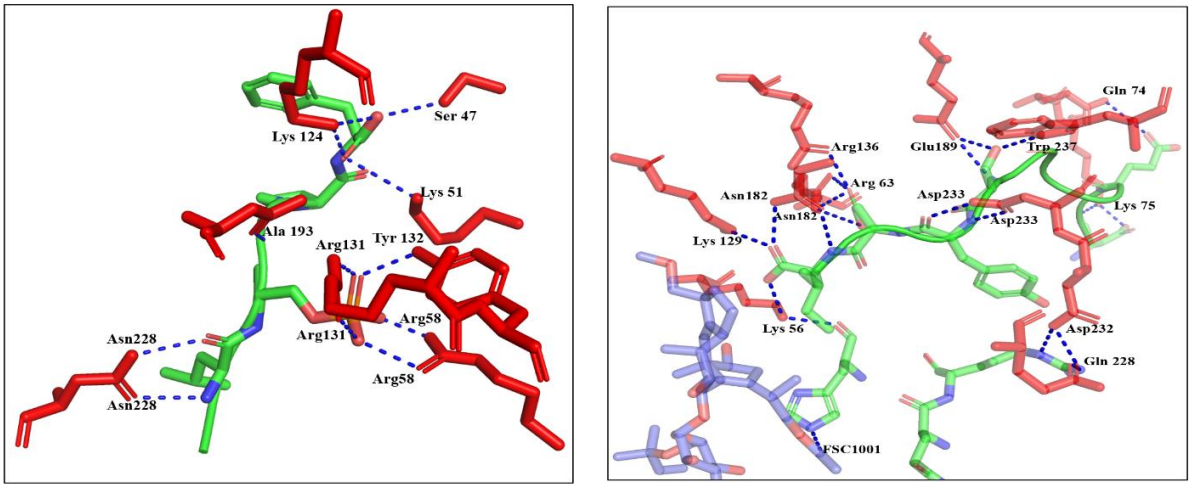
SNO	PROTEIN ORGANISM	NAME	UNIPROT ID	RESIDUE	CORRESPONDING P48348 RESIDUE	FUNCTION	BOUND PEPTIDE SEQUENCE	REFERENCES
1.	14-3-3 zeta (HS)	1QJB	P63104	LEU220	LEU229	Leucine residues within the α1 helix play a key role in phosphopeptide binding by forming a hydrophobic pocket. This pocket accommodates the side chains of specific residues in the bound peptides, enhancing the interaction and stability of the protein-peptide complex.	ARSHSYPA	Rittinger et al., 1999
				ASN173 ASN224	ASN182 ASN233	Asparagine residues in helices α1 and αg form hydrogen bonds with the NH and CO groups of the phosphopeptide backbone.These interactions stabilize the extended conformation of the bound peptide, enhancing its structural integrity.		
				LYS49 ARG56 ARG127 TYR128	LYS49 ARG63 ARG137 TYR137	Lysine, arginine, and tyrosine create a positive charge essential for interacting with the negatively charged phosphate group of		

						phosphoserine. This interaction stabilizes the peptide within the binding cleft.		
2.	14-3-3 EPSILON (HS)	2BR9	P62258	ARG57 ARG130 TYR131	ARG62 ARG136 TYR137	Arginine and tyrosine are essential for interacting with the phosphate group of the phosphopeptide, facilitating the binding of the 14-3-3 protein. Their interactions enhance the stability and affinity of this critical protein complex.	RRQRSAP	Yang et al.,2006
3.	14-3-3 (NP)	2O98	P93343	GLU19	GLU18	Lys943 in the peptide reduces the activity of the H+ ATPase enzyme. Replacing glutamic acid in 14-3-3 with lysine demonstrates their crucial role in binding to the peptide, highlighting the importance of Glu for effective interaction with H+ ATPase.	-	Ottmann et al.,2007
4.	14-3-3 (CP)	2O8P	Q5CYG0	ARG136 TYR137	ARG136 ARG137	Arginine and tyrosine are crucial for phosphopeptide binding, but in Cp14a, Arg68 is located farther away and oriented differently than the other residues,	-	Beokkx et al.,2011

						potentially impacting its binding efficiency.		
5.	14-3-3 (CP)	2NPM	Q5CUW0	ARG84 ARG157 TYR158	ARG62 ARG136 TYR137	The 14-3-3 protein was crystallised with the mode I peptide RAI(pS)LP, featuring a phosphorylated serine. Three key amino acids—arginine, arginine, and tyrosine are positioned to effectively bind and stabilise the phosphate group of the peptide.	RAISLP	Beokkx et al.,2011
6	14-3-3(OS)	3AXY	Q6ZKC0	ARG64, ARG129, TYR130	ARG62, ARG137, TYR137	These conserved residues form electrostatic and hydrogen bonds with the phosphate group of Hd3a (the florigen peptide), anchoring it in the amphipathic groove and ensuring specificity/stability of the complex.		Taoka et al., 2011
6.	14-3-3 (GL)	4ZQ0	E2RU97	ARG60 ARG135 TYR136 LYS53	ARG63 ARG136 TYR137 LYS56	Arginine, lysine, tyrosine, asparagine, and serine establish a robust network of hydrogen bonds with the phosphate group of phosphoserine in the peptide. This interaction enhances the stability and affinity of the peptide binding.	ARAASAPA	Cau et al.,2015
						The phosphate group of the phosphorylated		

7.	14-3-3 THETA (HS)	6BQT	P27348	ARG49 ARG56 ARG127	ARG56 ARG63 AGR136	amino acid interacts with basic residues in 14-3- 3θ (K49, R56, and R127) and forms a hydrogen bond with the hydroxyl group of Y128, enhancing the stability of the interaction.	DSYSNTLP VRKSVTPK NSYATTEN KTLPRSSS	Kast et al.,2019
8.	14-3-3 LAMBDA (AT)	8QT5	P48349	ASN233	ASN233	Asparagine, an amino acid, is known for its ability to form hydrogen bonds with the backbone of peptides.	LVGVTSSS CPADLTQ	Obergfell et al.,2024
				ARG136 TYR137	ARG136 TYR137	Arginine and tyrosine are important because, without these residues, 14-3-3 protein may lose its ability to effectively bind to peptides, impacting its biological activity and functionality.		

The fig 5 illustrate the conserved structural mechanism by which 14-3-3 proteins interact with phosphorylated peptides in plant systems. In A (PDB ID: 3AXY), the rice florigen Hd3a binds to the 14-3-3 protein through a phosphorylated motif, with residues like Lys124 and Arg131 forming hydrogen bonds that stabilize the complex an interaction crucial for regulating flowering. In B (PDB ID: 2O98), the 14-3-3 protein from *Nicotiana plumbaginifolia* interacts with the C-terminal region of H^+-ATPase, a key regulator of proton transport, using conserved basic residues such as Arg63 and Lys56 to recognize the phosphorylated peptide. Despite the different biological functions developmental signaling in A and membrane transport regulation in B both structures showcase a common recognition strategy where 14-3-3 proteins use positively charged residues to stabilize phosphopeptide binding. This highlights the central role of 14-3-3 proteins as versatile regulators in plant signaling networks.



A B

Fig 5. A Shows the interaction of rice florigen Hd3a with 14-3-3 protein and a phosphorylated peptide, where key residues like Lys124, Ser47, and Arg131 stabilize the complex, promoting flowering. B. depicts 14-3-3 from *Nicotiana plumbaginifolia* binding to the C-terminal peptide of H^+-ATPase, with Arg63, Lys56, and Glu189 involved in stabilizing the interaction, crucial

for proton pump regulation. Both highlight the essential role of 14-3-3 proteins in binding phosphopeptides through conserved basic residues.

6.2 Clonning and expression result

The gel electrophoresis results clearly demonstrate the successful execution of key molecular cloning steps. In Fig 6. the intact and prominent bands across all lanes verify efficient plasmid isolation without degradation. In B, the presence of two distinct bands in all samples following restriction digestion confirms both the integrity of the p Cold-TF vector and successful release of the 14-3-3 insert. This also suggests that the plasmids were correctly constructed and compatible with the NdeI and PstI restriction sites. C further validates that gel purification was successful, with clean, sharp bands of the expected sizes for both vector and insert. These results are crucial as they confirm that both DNA fragments are ready for downstream ligation and transformation steps, setting the foundation for the expression of the 14-3-3 protein in an appropriate host system.

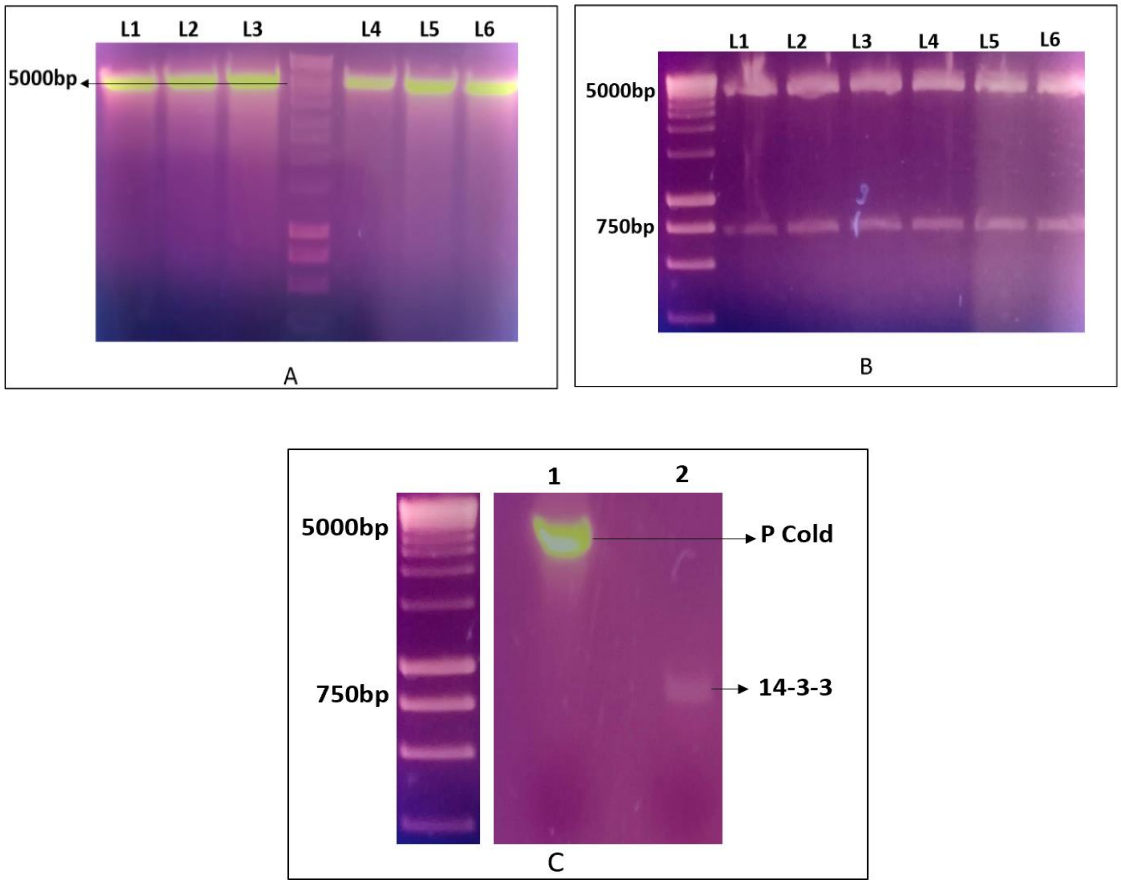


Fig 6. A Represents undigested p Cold-TF plasmid DNA isolated from six different clones (Lanes L1 to L3 and L5 to L6), showing a single, intact supercoiled band at approximately 5413 bp, with the molecular weight marker in Lane L4 used for size estimation. B Displays the double digestion of recombinant p Cold-TF plasmids using NdeI and PstI, where each lane (L1–L6) shows two distinct bands: a ~4654 bp fragment representing the digested p Cold-TF vector backbone and a ~759 bp fragment corresponding to the 14-3-3 gene insert, confirming the successful release of the target gene. C Shows the gel image after gel extraction, where Lane 1 exhibits a clear band at ~4654 bp (purified p Cold-TF vector), and Lane 2 shows a distinct band at ~759 bp (purified 14-3-3 gene insert), validating effective purification of both vector and insert for downstream applications.Agarose gel electrophoresis analysis used to confirm the digestion and purification of the p Cold-TF vector and the 14-3-3 insert**.**

To validate the transformation efficiency and confirm the successful uptake of the recombinant p Cold-TF 14-3-3 construct by *E. coli* BL21 cells, transformation was followed by plating on LB agar supplemented with ampicillin. The antibiotic acts as a selection marker to distinguish between transformed and untransformed cells. The results of this selection are shown in Fig 7 and 8.

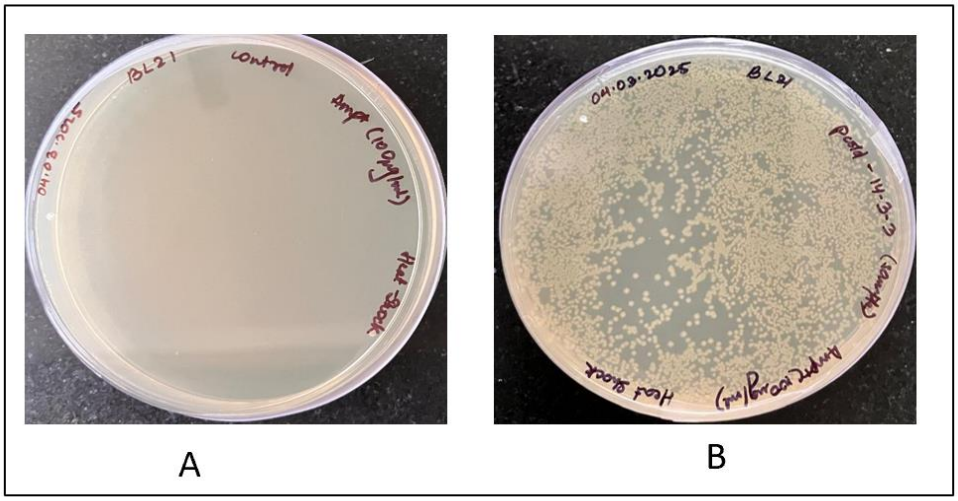


Fig 7. This image displays two LB agar plates supplemented with ampicillin (100 µg/mL) to assess the transformation efficiency of the p Cold-TF 14-3-3 construct into *E. coli* BL21 competent cells.

A (Control): This plate is labeled BL21 (control) and shows *E. coli* BL21 cells that were not given any plasmid. After heat-shock treatment, they were grown on an ampicillin-containing plate. No colonies are seen, which means the cells did not survive because they had no resistance to ampicillin. This proves the antibiotic worked properly and that only transformed cells can grow. B (Transformed Sample): This plate is labeled p Cold–TF 14-3-3 (sample) and shows many bacterial colonies. These are *E. coli* BL21 cells that were transformed with the p Cold-TF vector carrying the 14-3-3 gene. The presence of colonies shows that the cells

successfully took up the plasmid and became resistant to ampicillin. The even distribution of colonies suggests the transformation was efficient and the plating was done well.

This result confirms that the 14-3-3 gene was successfully inserted into the host cells. The transformed cells survived the antibiotic and can now be used for plasmid extraction, protein expression, and further experiments.

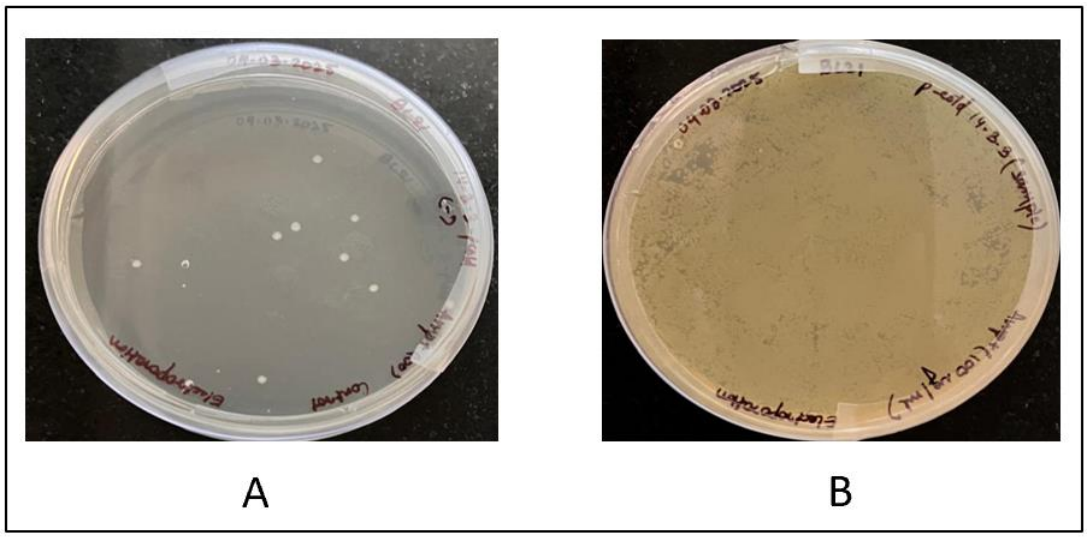


Fig 8. Comparison of control and transformed *E. coli* BL21 plates. Left Plate (Control): showing successful transformation with p Cold–TF 14-3-3 plasmid, while the control plate shows unwanted growth, indicating unreliable selection.

A (Control): This plate is labelled as the *control*, where *E. coli* BL21 cells without the recombinant plasmid were plated after heat-shock transformation. A few colonies are visible, which may be due to contamination or rare spontaneous resistance. However, compared to the test plate, the colony count is very low, confirming that most cells could not survive ampicillin due to a lack of the resistance gene. This plate helps confirm the selectivity of the antibiotic. B (Transformed Sample): This plate contains *E. coli* BL21 cells that were transformed with the p Cold–TF 14-3-3 plasmid. A thick layer of colonies is seen, which means the transformation

was successful. These cells have the ampicillin resistance gene and survive well on the selective plate. The high number of colonies shows that the transformation worked properly.

Since the control showed unwanted growth, we will not use it for further steps. Instead, we will proceed with the transformed sample from the heat-shock method for SDS-PAGE and protein expression studies, as it shows successful transformation and reliable results.

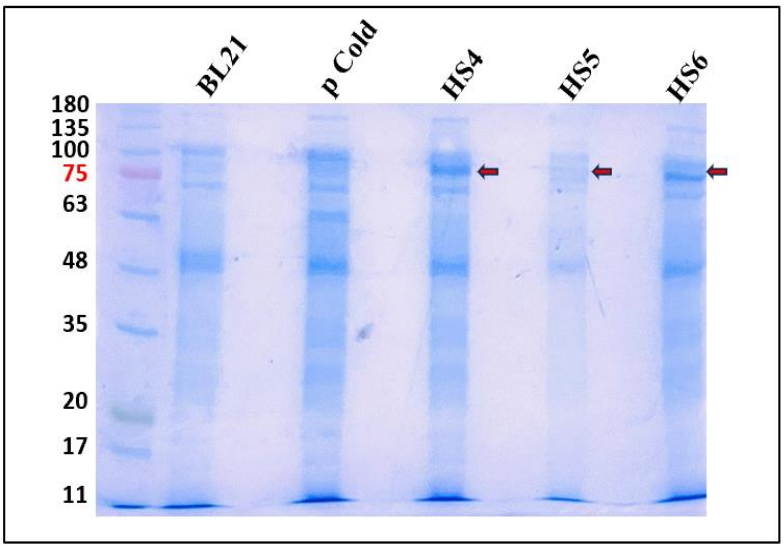


Fig 9. SDS-PAGE analysis of recombinant protein expression in *E. coli*. Lanes HS4, HS5, and HS6 (heat shock–transformed colonies) show a clear ~75 kDa band (red arrows), indicating successful expression of the recombinant protein. No such band is seen in the BL21 and pCold vector controls.

SDS-PAGE Analysis of Recombinant 14-3-3 Expression in *E. coli*

The SDS-PAGE analysis presented in Fig. 8 demonstrates the overexpression profile of the 14-3-3 recombinant protein with TF (~76.27 kDa) in using various induced colonies. Distinct bands corresponding to the expected molecular weight were observed prominently in colonies HS4 and HS6, as indicated by the arrows. Among them, HS4 displayed the most intense and well-defined band, followed by HS6, suggesting higher expression levels in these two colonies.

In contrast, HS5 showed a comparatively faint expression, while no visible expression was observed in the BL21 and p Cold-TF control lanes. Based on the clarity and intensity of the bands, colonies HS4 and HS6 will be selected for subsequent purification and downstream analyses.

7. Conclusion

This study investigated the structure, function, and expression of *Arabidopsis thaliana* 14-3-3κ protein, focusing on its potential role in calcium-mediated SOS signaling. The 14-3-3κ gene was successfully cloned into the p Cold-TF vector and overexpressed in *C* BL21 cells. SDS-PAGE analysis confirmed protein expression, with colonies HS4 and HS6 showing strong bands. Bioinformatic analysis identified conserved residues such as Arg, Lys, and Tyr, which are essential for phosphopeptide binding. Structural comparison using plant-derived PDBs (3AXY, 2O98, 1IBI) revealed different binding modes phospho-binding, active-site targeting, and groove binding but highlighted common basic residues (Arg, Lys) in all three. These residues play a critical role in stabilizing peptide-protein interactions. The presence of 14-3-3 binding motifs in the SOS1 C-terminal tail suggests possible regulation by 14-3-3κ, potentially influenced by calcium signaling. This supports the proposed role of 14-3-3κ in salt stress response and provides a foundation for future biochemical validation.

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