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IN SILICO CHARACTERIZATION OF PLANT SALT TOLERANCE PROMOTING KDP PROTEINS FROM *ALCALIGENES XYLOSOXYDANS*

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ABSTRACT

Soil salinity stress is a detrimental factor in crops production. Conventional methods of soil management and reclamation have been proved useless. On the contrary, exploiting the inherent genes and mechanisms of halotolerant bacteria can bring revolution in agriculture. Present study was designed to characterize ATPase dependent protein complexes kdpFABC and kdpDE in a salt tolerant bacterium *Alcaligenes xylosoxydans.* This complex enables plants to endure the saline environmental conditions through enhancing the K^+ ions influx. For characterization, protein sequences of three isoforms of kdpA, four of kdpB, two of kdpC and one of kdpE were retrieved from Uniprot database. These were analyzed via ProtParam tool, AlphaFold protein database and HDOCK server. Highest affinity for ATP molecule was observed in kdpB confirming its reported function of ATP hydrolysis. All documented proteins were found polar (except kdpE), alkaline (except one isoform of each kdpA and kdpB), thermostable, to exhibit complex 3D structure (except for kdpC and E) and *in vitro* stability. These properties of subunit proteins can be exploited to engineer the complex and produce osmotolerant transgenic plants.

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INTRODUCTION

Among the abiotic stresses, salinity is the most threatening and badly effects the sustainable productivity in agriculture (Safdar et al., 2019). Soil salinity is usually caused by accumulation of sodium (Na^+) and chloride (Cl^-) ions (Etesami and Glick, 2020). Literature has reported 70% reduction in yields of *Hordeum vulgare*, *Zea mays*, *Oryza sativa* and *Triticum aestivum* crops due to salinization (Etesami and Glick, 2020). It causes an imbalance in plant growth hormones (Seymen et al., 2023), nutrient immobilization, an increase in toxic ions and reactive oxygen species (ROS) (Faisal et al., 2023), a reduction in stomatal conductance, a decrease in photosynthesis rate and carbon dioxide (CO_2) production (Seymen et al., 2023), senescence of leaves (Moreira et al., 2023), a decrease in the diversity of the soil microbiome (Zhang et al., 2023), loss of turgidity (Dourado et al., 2022), a decrease in osmotic potential, increased plant synthesis of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) (Semida et al., 2021), glutathione reductase (GR), catalase (CAT), and ascorbate peroxidase (APX) (Sofo et al., 2015), and promotes the accumulation of osmolytes, including betaine, glycine, and proline (Hernandez-Leon and Valenzuela-Soto, 2023). Additionally, it enhances the production of abscisic acid (ABA), ethylene, and jasmonic acid (JA) hormones (Mishra et al., 2021; Ullah et al., 2021). Numerous precautionary measures have been implemented to address the menace of salinity stress in plants, such as the reclamation of saline soils, the

assessment of salt tolerance potential in plants through the analysis of physiological and morphological characteristics, growth rate, and salt influx mechanisms (Ashraf and Munns, 2022). However, these conventional approaches have failed to yield positive results (Berg, 2021; Daniells et al., 2001). Consequently, the production of transgenic plants, initially through selective breeding and later via genetic engineering, has proven to be an emerging and alternative solution to salt stress (Etesami and Glick, 2020; Kashyap et al., 2021; Shelake et al., 2022). Several halotolerant bacteria have been isolated and characterized to explore their innate mechanisms and genes associated with adaptations in a saline environment (Pourbabaee et al., 2016; Kerbab et al., 2021; Kumawat et al., 2022).

Several halotolerant bacteria that contribute to plants' endurance under salinity stress have been reported in the literature. These include *Paenibacillus polymyxa* YC0136 (Li et al., 2021), *Erwinia gerundensis* (Saldierna Guzmán et al., 2021), *Delftia acidovorans* RAY209 (Suchan et al., 2020), *Bacillus megaterium* IDE-14, *Pseudomonas putida* IDE-01, and *Azospirillum brasilense* IDE-06 (García et al., 2022), *Enterobacter roggenkampii* ED5 (*Guo et al., 2020*), *Cronobacter muytjensii* JZ38 (Eida et al., 2020), *Jejubacter calystegiae*, *Pseudomonas thivervalensis* SC5 (Nascimento et al., 2021), *Pantoea agglomerans* ANP8 (Noori et al., 2021), *Bacillus paralicheniformis* ES-1 (Iqbal et al., 2023), *Brevibacterium sediminis* MG-1 (Lutfullin et al., 2022), *Bacillus* sp. BH32 (Belaouni et al., 2022), *Stenotrophomonas rhizophilia* IS26, and *Stenotrophomonas* 169 (Ulrich et al., 2021), *Escherichia coli* (Barut et al., 2023), and *Achromobacter xylosoxydans* (Jana and Yaish, 2021).

These bacteria have been investigated at the genetic level, leading to the identification of a large number of genes associated with halotolerance. These include groES and groEL (Patel et al., 2018), dnaJK, htpGX, grpE, clpB, clpX, and clpE (Yu et al., 2021), dnaK and dnaJ (Zhichang et al., 2010), groL, groS, and hspQ (Xia et al., 2023), ibpA, clpXP, and ibpB (Horváth et al., 2008), clpC (Chao et al., 2005), htpX (Liu et al., 2016), AtTPK3 (Carraretto, 2013), mrpABCDEFG (Suarez et al., 2019), kdp operon (Liu et al., 2016), MRP (Cui et al., 2020b), czcD and czcR (Asaf et al., 2020), copD and rcnA (Girma et al., 2020), and yjbQ (Gilhar et al., 2022).

During high salinity conditions, the ion homeostasis of plants is mainly disturbed due to the inward movement of Na+ ions and outward movement of K+ ions (Demidchik et al., 2002; Mäser et al., 2002; Khan et al., 2020). High cytoplasmic K+ ion concentration plays a crucial role in the recovery of flora from salt stress (Wu et al., 2018; Khan et al., 2020; Kumari et al., 2021). Hence, the proteins associated with K+ ion transport are most important among all the proteins reported so far (Szczerba et al., 2009). One of these is known as kdp ATPase, which is a high-affinity K+ transport system (Pedersen et al., 2019). The kdp is a P-type ATPase that contributes to K+ ion intake, and this K+ movement is coupled to ATP hydrolysis (Chen et al., 2021).

It is a complex system in salt-tolerant bacteria encoded by a polycistronic operon, kdpFABC, comprising of four subunits encoding structural genes. Expression of these structural genes depends on another operon, kdpDE. The kdpFABC and kdpDE are collectively called the kdp regulon (Muntyan et al., 2020). The kdpA, kdpB, kdpC, kdpD, kdpE, and kdpF are associated with K+ binding/transport, ATP hydrolysis, catalytic chaperone activity, structural integrity, sensor kinase activity, and response regulation, respectively (Kixmüller, 2011). This system has been identified in several halotolerant bacteria such as *Halobacterium salinarum* R1, *Halomonas elongata*, *Bacillus* sp. BH32, *Enterobacter* sp. SA187, *E. roggenkampii* ED5, and *Alcaligenes xylosoxydans* (Kunte, 2005; Strahl and Greie, 2008; Andrés-Barrao et al., 2017; Guo et al., 2020; Jana and Yaish, 2021; Belaouni et al., 2022).

Considering the physiological role of K+ ions in plant growth and the significance of the kdpFABC operon in adapting to salinity stress (Heermann et al., 2009; Pedersen et al., 2019; Cui et al., 2020a), the present study has been initiated. We have characterized the subunits of the kdpFABC and kdpDE complexes, which are encoded by the kdpA, kdpB, kdpC, and kdpE genes in *A. xylosoxydans*. This characterization may be helpful in exploiting this bacterium for the production of transgenic salt-tolerant plants. The tools used for characterization in the present study are highly significant for gaining insight into the structural and functional features of the protein for downstream transgene development (Pramanik et al., 2017; Bhattacharya et al., 2018; Dutta et al., 2018).

MATERIALS AND METHODS

Uniprot database

Uniprot database (available at https://www.uniprot.org,

accessed on September 2023) was consulted to retrieve the sequences of protein isoforms of kdpA, kdpB, kdpC and kdpE. The primary accession numbers of the proteins documented in present study are A0A176XW07 and A0A424WI79 for kdpA, A0A1R1JM35, E3HLY0, A0A424WIA5 and A0A176XWB1 for kdpB, A0A1R1JM24, A0A424WI81 and E3HLX9 for kdpC and A0A0D6GCH6 for kdpE (Supplementary data Table 1).

ProtParam

These sequences were analyzed through ProtParam tool (available at https://web.expasy.org/protparam/, accessed on September 2023) and physiological properties of present study proteins were determined. Properties included the number of amino acids, molecular weights, aliphatic index, instability index, half-life, isoelectric point (pI) and grand average of hydropathy (GRAVY).

AlphaFold

To predict the 3D configuration of kdp subunit proteins, AlphaFold Protein Structure Database (available at https://alphafold.ebi..ac.uk, accessed on September 2023) was used.

HDOCK server

In order to determine the affinity of all the protein components of kdpFABC and kdpDE complexes with ATP, docking was performed using HDOCK server (hdock.phys.hust.edu.cn, accessed on September 2023). To perform docking the pdb files of proteins were retrieved from AlphaFold database. The 2D SDFfile for ATP molecule was obtained from ZINC database (http://zinc.docking.org, accessed on September 2023). This SDF file was converted into pdb format via an open source chemical tool box, Open Babel (http://openbabel.org). The pdb files were uploaded to HDOCK server to perform docking analysis.

RESULTS

Present study analyzed the subunit proteins of kdpFABC and kdpDE complexes i.e. kdpA, kdpB, kdpC and kdpE from *A. myxosoxydans* at different levels i.e. physiological properties, 3D configuration and ATP binding tendency.

Prediction of physicochemical properties

Physicochemical properties of proteins documented in present study were predicted using ProtParam tool (Table 1). The molecular weight ranged between 77089.57 and 20608.58 for kcpB (A0A176XWB1) and kdpC (A0A424WI81), respectively. Half-life remains unchanged in all the cases i.e. > 10. Variation was observed in values of pI. i.e. 6.61 to 7.70 for kdpA, 6.96 to 8.77 for kdpB, 9.15 to 9.85 for kdpC and 6.08 for kdpE. Highest instability index was recorded to be 42.42, 38.99 and 38.33 for kdpE and isoforms of kdpC, respectively. On the contrary, kdpA and kdpB exhibited the least values (29.95 to 34.32). Aliphatic index values ranged between 99.21 and 110.67. Lowest value of GRAVY was observed for kdpE i.e. -0.103 and highest in kdpA (0.615 to 0.659).

Table 1: Prediction of physicochemical properties of protein isoforms kdpA, kdpB, kdpC and kdpE of kdpFABC and kdpDE complexes using ProtParam tool.

Sr. No.	Accession ID	Mol. Wt.	Isoelectric point (pI)	Half-life (hr)	Instability index	Aliphatic index	GRAVY
			kdpB				
1	A0A1R1JM35	75313.79	8.42	> 10	29.95	111.61	0.410
2	E3HLY0	74985.22	7.76	> 10	30.88	110.26	0.427
3	A0A424WIA5	74958.18	6.96	> 10	31.22	110.67	0.431
4	A0A176XWB1	77089.57	8.77	> 10	34.32	109.72	0.416
			kdpA				
5	A0A1R1JM24	62363.04	7.01	> 10	31.53	109.57	0.615
6	A0A176XW07	61485.98	7.70	> 10	30.99	110.03	0.659
7	A0A424WI79	62455.03	6.61	> 10	31.99	109.83	0.622
			kdpC				
8	A0A424WI81	20608.58	9.15	> 10	38.33	99.21	0.170
9	E3HLX9	20811.88	9.85	> 10	38.99	99.70	0.176
			kdpE				
10	A0A0D6GCH6	25624.37	6.08	> 10	42.24	102.22	-0.103

Prediction of 3D configuration

The 3D structures of kdpFABC and kdpDE complexes proteins predicted using AlphaFold are shown in Figure 1. Different colors are showing per residue model confidence scores (pLDDT) of the model. i. e. **dark blue** (very high pLDDT > 90), **sky blue** (High 90 < pLDDT > 70), **yellow** (Low 70 < pLDDT > 50), **orange** (very low pLDDT < 50).

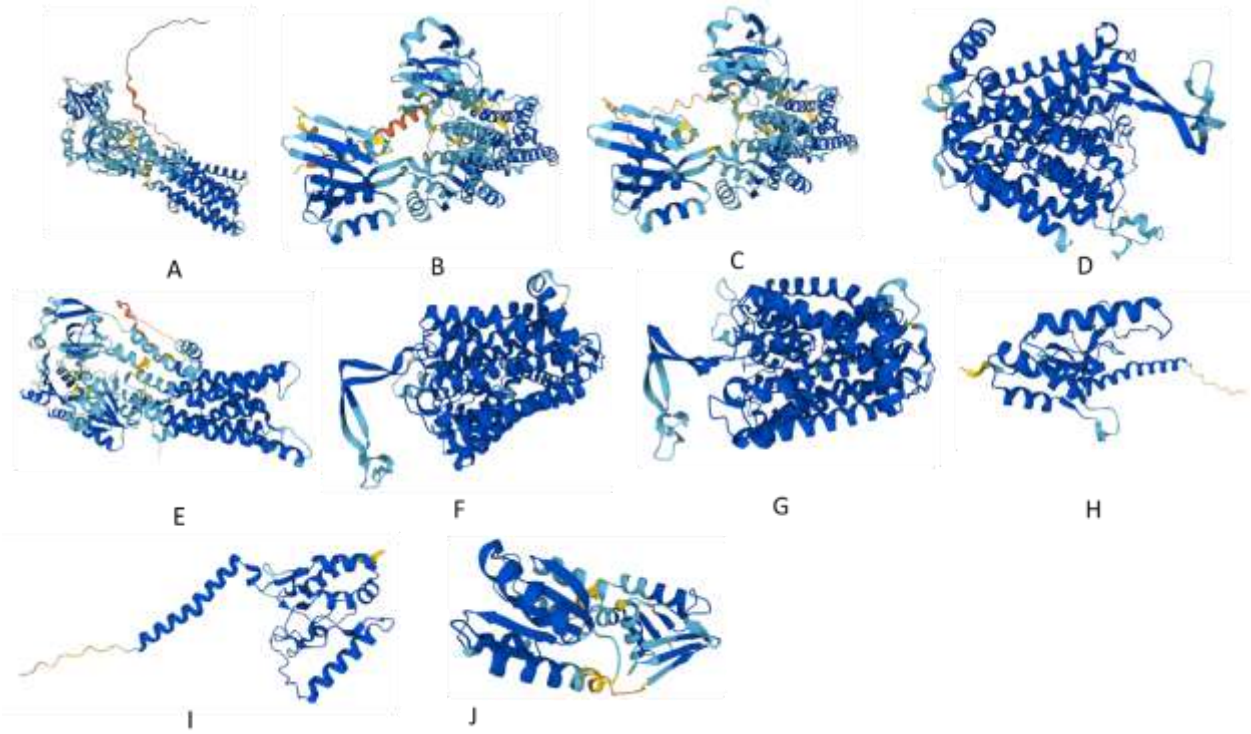

Figure 1: Prediction of 3D configuration of protein isoforms kdpA, kdpB, kdpC and kdpE of kdpFABC and kdpDE complexes documented in present study, using AlphaFold protein database.
Different colors are showing per residue model confidence scores (pLDDT) of the model. i. e. **dark blue** (very high pLDDT > 90), **sky blue** (High 90 < pLDDT > 70), **yellow** (Low 70 < pLDDT > 50), **orange** (very low pLDDT < 50).

Among the isoforms of kdpA, A0A424WI79 and A0A176XW07 exhibited similarity in configuration, however, a slight variation was observed while the kdpA variant with accession ID A0A1R1JM24 was considerably different from the other two variants. In case of kdpB isoforms, variants with IDs E3HLY0 and A0A424WIA5 exhibited structures with considerable similarity while isoforms A0A176XWB1 and A0A1R1JM35 were different from each other and also from the other closely similar variants. The kdpC isoforms A0A424WI81 and E3HLX9 were diverse from each other. These two and kdpE exhibited less complex structures than kdpA and kdpB.

Prediction of ATP binding affinity

The binding energies, confidence scores and Ligand rmsd of present study proteins with ATP molecule were predicted (Figure 2 and Table 2).

DISCUSSION

The literature has reported various adaptations in plants to cope with salinity problems, such as association with different halophilic bacteria and the production of various secondary metabolites. Different genes from salt-tolerant bacteria enable plants to survive this stress. Additionally, several metabolites have been reported to be produced in plants in response to salinity, including choline O-sulfate, proline, glycine, betaine, dimethylsulfoniopropionate, mannitol, trehalose, fructose, galactose, sorbitol, phenolics, and dimethyl inositols. These secondary metabolites help plants survive through detoxification and osmotic activities, as well as through enzyme activation and inactivation (Wang et al., 2016; Liu et al., 2017; Gul et al., 2022).

Table 2: Prediction of binding tendency of protein isoforms kdpA, kdpB, kdpC and kdpE of kdpFABC and kdpDE complexes, with ATP molecule using HDOCK docking server.

Sr. No.	Docking score	Confidence score	Ligand rmsd (Å)
		kdpB	
1	-179.84	0.6449	52.37
2	-179.23	0.6421	52.65
3	-209.67	0.7673	41.40
4	-171.91	0.6078	52.43
		kdpA	
5	-171.50	0.6059	30.56
6	-174.88	0.6219	30.29
7	-179.61	0.6439	26.20
		kdpC	
8	-167.42	0.5862	45.40
9	-168.42	0.5911	42.91
		kdpE	
10	-188.58	0.6839	45.68

Highest binding affinity was observed in kdpB isoform A0A424WIA5 with docking score of -209.67. The lowest affinity was observed to be -171.50 for kdpA isoform with ID A0A1R1JM24.

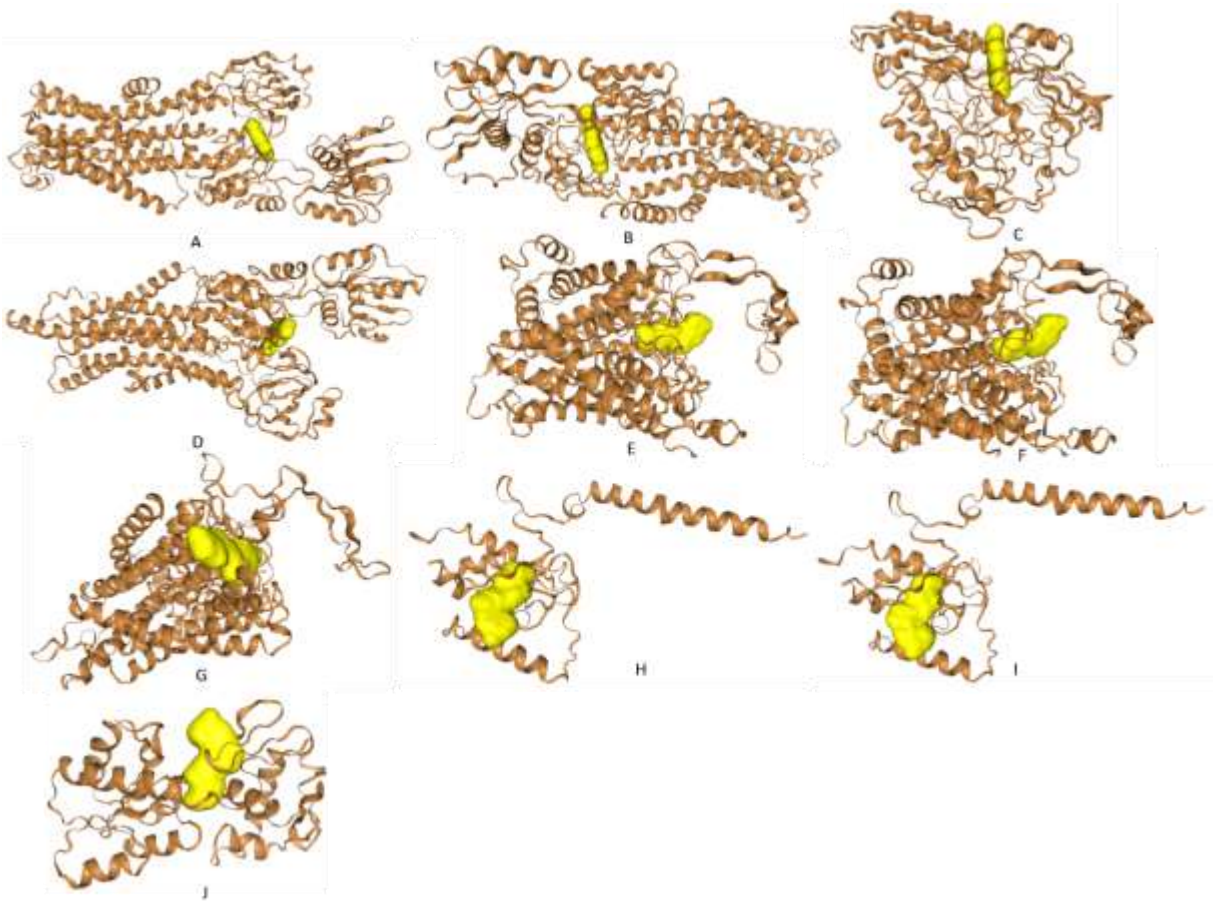

Figure 2: Prediction of docking scores reflecting binding affinities of protein isoforms kdpA, kdpB, kdpC and kdpE of kdpFABC and kdpDE complexes, with ATP molecule, using HDOCK server.

Present study deals with characterization of the kdpFABC complex in the halotolerant bacterium *A. xylosoxydans*. Multiple studies identified the presence of kdp ATPase-encoding genes in many halotolerant bacteria, but no one has characterized this complex protein (Gumulya et al., 2018; González-Rosales et al., 2022; Najjari, 2023). Therefore, this is the first-ever study reporting the characterization of proteins constituting the kdp ATPase complex. This complex comprises of kdpA, kdpB, kdpC, kdpD, and kdpE protein components. The kdpA, B, and C are structural, while kdpD and E are regulatory in function. The kdpD serves as a sensor and perceives the stimulus, while kdpE regulates the response to this stimulus (Kixmüller, 2011).

In a single bacterium, genes constituting the kdpFABC operon may encode different isoforms of the same protein. We have compared three isoforms of kdpA, four isoforms of kdpB, and two isoforms of kdpC. The comparison was based on physicochemical attributes, 3D structure, and binding tendency with the ATP molecule.

To logically and precisely design a protein at the molecular level, it is important to select genes for cloning purposes that impart the best physicochemical attributes. These properties will be helpful in designing an operon for cloning purposes and will also provide clues for the conditions that can be applied for the purification of the recombinant protein from transgenic plants (Tandang-Silvas et al., 2011). These properties may also contribute to improved protein-protein interactions (Banerjee et al., 2015).

The pI value indicates the acidic or alkaline nature of proteins. All the subunits of the kdp complex characterized in the present study were found to be alkaline except for one isoform of kdpA (A0A424WI79) and kdpB (A0A424WIA5). The pI is the point of least solubility of a protein. Computing the pI values can be helpful in selecting the buffer pH at the time of protein purification via crystallization. The use of a buffer with a pH equal to or closer to the pI will lead to effective protein crystallization (Kantardjieff and Rupp, 2004).

Additionally, the aliphatic index is a measure of the thermostability of a protein as it indicates the proportion of aliphatic amino acids. The values for all the kdp subunits are quite high (ranging from 99.21 to 111.61), corresponding to their increased thermostability (Ikai, 1980). The instability index is directly related to *in vitro* protein stability. Except for kdpE, all the subunit proteins of the kdp complex exhibited instability index values below 40, corresponding to their increased *in vitro* stability (Gamage et al., 2019). The GRAVY values of proteins indicate their hydrophobicity and hydrophilicity. In present study, all the documented proteins were found to be polar with positive values, except for kdpE (Kyte and Doolittle, 1982).

The 3D configurations predicted in present study revealed diversity among the protein isoforms encoded by the same gene. The kdpB domain, associated with ATP hydrolysis, exhibited a highly complex configuration in all three isoforms. Similarly, two isoforms of kdpA, associated with K+ binding/transport, also demonstrated highly complex structures. Simplest structures were observed in the kdpC isoforms and kdpE. KdpC is associated with catalytic chaperone activity, accelerating protein folding by providing the activation energy (Irzik et al., 2011). KdpE modulates the response of the entire kdp complex. The major functions of this complex are actually performed by kdpA and kdpB, which have a complex level of folding. The complexity observed in different parts of the kdp complex justifies their functions.

According to the literature, in the kdpFABC and kdpDE complex, kdpB is the energy provider and performs ATP hydrolysis (Bramkamp and Altendorf, 2004). Our findings from the docking analysis are in accordance with this, as the highest binding affinity for ATP molecule was observed in the case of kdpB isoform, which had the highest docking score, i.e., -209.67.

CONCLUSION

Designing a project for transgenic crop development requires knowledge of the gene of interest. Characteristics of the kdpFABC complex determined in this project may assist in the selection of genes with the best functioning. An artificial operon encoding the best version of the kdpFABC complex may be designed and inserted into plants to induce osmoadaptation.

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AUTHOR CONTRIBUTION

FM perceived the idea, designed methodology, retrieved

data from database, performed analysis, wrote up of manuscript and proofread it.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The sequences of proteins documented in present study are available at Uniprot database (https://www.uniprot.org).

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