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CHANGES IN ENDOGENOUS LEVELS OF IAA AND PAA IN RICE SEED DURING DEVELOPMENT, PREDICTION OF AUXIN SIGNALING PROTEINS AND DOCKING FOR ITS BINDING EFFICIENCY WITH AUXIN MOLECULES

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ABSTRACT

Indole-3-acetic acid (IAA) and phenyl acetic acid (PAA) are two major physiologically active auxins, found in higher plants. The objective of this study was to evaluate changes in the endogenous levels of IAA and PAA in rice (*Oryza sativa*) seed and predict probable transporter and efflux (signaling) proteins for docking with auxins from the available rice genome data. IAA and PAA were estimated during rice seed development using sensitive and specific antibodies against each hormone. The levels of IAA and PAA were higher when sink size development was at its peak, suggesting an important role played by the auxin in sink size development of rice seed. Furthermore, auxin binding transporters proteins of rice were confirmed for docking purpose and compared with *Arabidopsis* and maize proteins. In this context, PAA docked with known IAA proteins; although, docking score of IAA was higher than that of PAA in rice seed. Twelve genes of the auxin efflux carrier (PIN family) of rice were also analysed for phylogeny relatedness with *Arabidopsis* and maize, to further understanding auxin concentrations and efflux within rice seeds. The predicted PIN proteins for IAA efflux of rice showed docking affinities with PAA, revealed its role in maintaining physiological concentrations of auxins in rice seeds.

Key Words: Immunoassay, Indole-3-acetic acid, *Oryza sativa*, phenyl acetic acid

RÉSUMÉ

L'acide indole-3-acétique (IAA, Indole-3-acetic acid) et l'acide phénylacétique (PAA, phenyl acetic acid) sont deux auxines physiologiquement actives majeures, trouvées dans les plantes supérieures. L'objectif de cette étude était d'évaluer les changements dans les niveaux endogènes d'IAA et de PAA dans les graines de riz (*Oryza sativa*) et de prédire les protéines de transport et d'efflux (signalisation) probables pour l'amarrage aux auxines à partir des données disponibles sur le génome du riz. L'IAA et le PAA ont été estimés au cours du développement des semences de riz en utilisant des anticorps sensibles et spécifiques contre chaque hormone. Les niveaux d'IAA et de PAA étaient plus élevés lorsque le développement de la taille du puits était à son maximum, suggérant un rôle important

joué par l'auxine dans le développement de la taille du puits des graines de riz. De plus, les protéines transporteuses de liaison aux auxines du riz ont été confirmées à des fins d'amarrage et comparées aux protéines d'*Arabidopsis* et de maïs. Dans ce contexte, le PAA s'est amarré à des protéines IAA connues ; cependant, le but d'amarrage de l'IAA était plus élevé que celui du PAA dans les semences de riz. Douze gènes du transporteur d'efflux d'auxine (famille PIN) du riz ont également été analysés pour la phylogénie avec *Arabidopsis* et le maïs, afin de mieux comprendre les concentrations d'auxine et l'efflux dans les graines de riz. Les protéines PIN prédites pour l'efflux d'IAA du riz ont montré des affinités d'amarrage avec le PAA, ont révélé son rôle dans le maintien des concentrations physiologiques d'auxines dans les graines de riz.

Mots Clés: Immunoessai, acide indole-3-acétique, *Oryza sativa*, acide phénylacétique

INTRODUCTION

Auxins play a decisive role in almost all processes of plant cell growth and development. Auxin molecules are known to regulate cell division (Tank *et al.*, 2014), apical dominance (Reinhardt *et al.*, 2002), lateral root formation (Hidehiro *et al.*, 2007), primordia formation in leaf and flowers (Dubrovsky *et al.*, 2008), cell elongation (Gokani and Thaker, 2002), and sink size development (Chudasama and Thaker, 2007). In all these, the physiological concentration of auxin is the most important element for the effective functions of the cell. Although, IAA is the most studied auxin in plants, other molecules that possess auxin activities are also widely studied; namely PAA (Wightman and Lightly, 1982), Indole-3-butyric acid (IBA) (Sauer and Kleine-Vehn 2011) and 4-Chloroindole-3-acetic acid (4-cl-IAA) (Reinecke, 1999). In addition, naturally present coumarin acetic acid (CAA) was also demonstrated to possess auxin activities in *in vitro* grown cotton fiber (Barai *et al.*, 2005).

Auxin status in the cell is reported as (i) free form which is readily available for growth; (ii) conjugated form this released as and when required by the cell for metabolism; otherwise remained stored (Gokani and Thaker, 2002; Chudasama and Thaker, 2006/7); and (iii) the oxidised form as a result of IAA oxidase or peroxidases activities, participate in cessation of growth (Thaker, 1998). The concentration of free forms depends upon the rate of

synthesis (Thaker *et al.*, 1986), transport from cell to cell (Nakum *et al.*, 2013) and availability of the receptor molecules (Trevawas, 1982). The concentration of the conjugated forms depends on the availability of conjugating molecules in the cell and oxidised form regulated by the enzyme activities of auxin oxidases (Thaker, 1998).

Physiological functions of the auxins are regulated by a suite of protein molecules that participate in auxin influx at the apex of the cell, to efflux from the base to the next cell of the plant (Trevawas, 1982). Formation of protein-protein complex and its turnover is a key point in auxin signaling. Two major groups of proteins participate in auxin functions; namely (i) auxin binding and transporter proteins; and (ii) efflux transporter proteins. Furthermore, auxins are perceived by two class of receptors, namely the ABP1 extracellular receptor (Xu *et al.*, 2014), and intracellular TIR1/AFB class receptors (Mockaitis and Estelle, 2008).

Four auxin signaling orthologous genes for TIR1 are present in the rice genome (<https://rgp.dna.affrc.go.jp/index.html>; <https://www.ncbi.nlm.nih.gov>); however, their protein structure is not known. Furthermore, ASK1 and cullin1 (Cul1) were reported to form a complex with TIR1, which regulates IAA signaling in *Arabidopsis* (Dharmasiri *et al.*, 2005a; 2005b). But molecules transporting PAA are missing in the literature for any plant. The proteins of *Arabidopsis* can be used as

model to predict proteins structure in rice and to understand its probable function in auxin efflux/influx for IAA and PAA.

Auxin efflux is regulated by the second set of proteins known as the Polarised auxin transport (PIN) family (Køeëket *et al.*, 2009). A total of 12 such PIN proteins identified in the higher plants has a role in auxin efflux in the cells. In *A. thaliana*, 8 PIN proteins are reported. In maize and rice, 12 PIN proteins are present and from these, PIN9 and PIN 10 are reported only in monocots (Cristian *et al.*, 2012).

Rice (*Oryza sativa*) is an important cereal crop grown worldwide for human consumption. Development of rice seed on panicle from the day of flowering to maturation passes through the series of metabolic events, which are regulated by the hormones. Auxins present in the developing seed cell participate in cell division and cell elongation, which finally determine seed size (Bhatt and Thaker, 2008, Chudasama and Thaker, 2008). Estimation of endogenous hormones is technically difficult because they are synthesised in minute quantities; as low as 10^{-5} M concentration (Trevawas, 1982). Immunoassays are sensitive and reliable for accurate estimation at low concentrations (Weiler *et al.*, 1981), and therefore, an attempt has been made to evaluate endogenous concentrations using antibodies raised against each, IAA and PAA. It is presumed that changes in IAA and PAA levels will help to understand the role of these molecules in the rice seed development.

To understand the molecular basis of physiological responses of auxin in tissues containing different levels of auxins, it is important to know the auxin sensitivities to TIR1 and ABP1 proteins in rice. The compact nature of the rice genome provides a distinct advantage in gene isolation and genomic sequencing, and thus the rice species has been selected as a target species for genome research by a number of research groups

worldwide (Wang *et al.*, 2009; Chen *et al.*, 2014). The objective of this study was to investigate the endogenous IAA and PAA from rice seed and auxin binding capacities with transporter and efflux protein.

MATERIALS AND METHODS

Plant sampling and hormone assay. Rice (*Oryza sativa*), varieties GR-11 was grown at the ASPEE research foundation, TANSA Farm, in Mumbai (19° 11' 299188'' N, 72° 50' 308076' E) in India. The cultural practices including irrigation, application of fertilisers, insecticides and pesticides were conducted as normal field practices. From the day of anthesis, uniformly growing panicles in the field were tagged to minimise variations in sampling (Jhala and Thaker, 2015) and harvested every day from day 1 until maturity for hormone analysis.

Estimation of hormones. Seeds (approx:150-500 mg) of different ages were crushed with liquid nitrogen and mixed with extraction solution containing 5 ml of 80% methanol (v/v) and 100 mg L⁻¹ ascorbic acid as an antioxidant. The mixture was stirred at 100 rpm using a magnetic stirrer and incubated for 48 hr at 4°C in dark. The mixture was centrifuged at 10,000 g for 10 minutes at 0°C, to collect supernatant. Its pellets were washed twice in extraction solution and the pooled supernatant was concentrated using lyophiliser. These extracts were used as sample sources of hormones for estimation, after partial purification on PVPP column as described by Gokani and Thaker (2002).

Estimation of IAA and PAA concentrations. Endogenous levels of IAA and PAA were estimated using specific antibodies against each by a comparatively more sensitive and specific technique, i.e. indirect ELISA, standardised using internal standards and second rabbit IgG tagged with peroxides (Gokani and Thaker,

2002). In a nut shell, PGR-casein conjugate (300 µl) was coated on ELISA plate and incubated for overnight at 4 °C. This was followed by washing with PBS-T, and then by blocking of free protein binding sites of well with egg albumin and incubated for 1 hr at 37 °C. Antibodies against PGRs (either IAA or PAA) mixed with samples separately for the each data points and were coated and incubated for 3 hr at 37 °C. Finally, the plate was coated with anti-Rabbit IgG, tagged with peroxidase and the colour was developed using O-phenylenediamine as a substrate. The reaction was terminated by addition of 6N sulfuric acid (50 µl). After each coating, the ELISA plate was washed thoroughly with PBS containing 0.05 % Tween-20. The colour developed was measured at 490 nm by ELISA Reader (µ Quant, Biotek, USA). The assay for each hormone was performed thrice and the respective mean values were calculated.

Raising of antibodies. To raise antibodies against IAA and PAA, IAA-BSA (Weiler, 1981) and PAA-BSA (Gokani and Thaker, 2002) conjugates were prepared. Antibodies raised earlier (Patel and Thaker, 2007) were purified for IgG using DEAE cellulose chromatography. The purified antibodies were lyophilised and stored at -20 °C, until when it was used. For estimation of IAA and PAA, each hormone conjugate was prepared with casein instead BSA to avoid cross reactivity of the conjugated protein (Gokani and Thaker, 2002).

Calibration for IAA and PAA. A calibration curve prepared for IAA and PAA showed a linear relationship with concentration, in the range 50-400 and 25-250 ng per well, for IAA and PAA, respectively. For preparation of the calibration curve, standards of 25-500 ng per well, were mixed with an optimum dilution of antibodies (1:10,000) instead of samples prior to coating on the plate. Dilution of samples and addition of internal standard to test the sensitivity of the assay was done in this

calibration range. With each plate, at least 3-5 known concentrations of either IAA or PAA were measured as standard for calibration for assay validation to avoid experimental errors. Approximately 20 samples in triplicates were assayed per plate; while the remaining wells of the 96 well plate contained positive controls (known concentration of auxin), negative controls (only reaction mixture) and randomly selected samples with internal standard were also included. All estimations were done in triplicates.

Statistical analysis. Data for the hormone concentrations were taken in triplicates and the mean values calculated. The samples with internal standard and calibration were used to validate the assay from each plate. As such, samples with and without internal standards were calculated. By using calibration value, each data point was calculated. A P-value of 1% or less were considered significant for data interpretation.

***In silico* analysis of signaling molecules.** *In silico* work were categorised in three parts as below

- (i) Auxin receptor proteins prediction form rice sequences:

Known auxin binding and signaling proteins structure were derived from PDB (<https://www.rcsb.org/>) i.e. from *Zea mize* and *Arabidopsis thaliana* respectively. Auxin binding structure and signaling proteins were pBLAST in UNIPROT (<https://www.uniprot.org/>). UNIPROT ID with maximum BLAST scores in rice used for Rice Protein prediction study. Uniprot databases containing protein sequences of SKP1 (UniProtKB: Q6PL11) and TIR1 (UniProtKB: Q0DKP3), were used for prediction of rice proteins; UniProtKB accession code of the target protein as

input for modeling. The retrieved sequences of rice were used for protein prediction in SWISS Model (<https://swissmodel.expasy.org/>).

- (ii) Docking of auxin receptor proteins of *Arabidopsis thaliana*, maize and rice with auxins (IAA and PAA):

Predicted proteins of rice were confirmed using Protein Structure Comparison Tool V.3.0.8 (jCE V.1.1). Molegro Virtual Docker program has been used for docking of known and predicted proteins with IAA and PAA molecules.

- (iii) Multiple sequence alignment and phylogenetic analysis of auxin efflux carriers and membrane prediction:

To perform a phylogenetic analysis of Polarised Auxin Transport (PIN) proteins sequences, we collected sequences from <https://www.uniprot.org> of rice, *Zea mays* and *Arabidopsis* sequences. Uniprot ID of *Oryza sativa*. UPGMA Phylogenetic analysis work had been performed in CLC workbench-5 to know the relationship between *A. thaliana* (dicot), maize and rice (monocot) PIN family proteins.

The HMMTOP (Hidden Markov Model for Topology Prediction) transmembrane topology prediction server (<http://www.enzim.hu/hmmtop/>) was used for rice PIN sequences OsPIN1a, OsPIN1b, OsPIN1c, OsPIN1d, OsPIN2, OsPIN3a, OsPIN3b, OsPIN5a, OsPIN5b, OsPIN5c, OsPIN8 and OsPIN9 membrane prediction.

RESULTS AND DISCUSSION

To understand the role of auxins in rice seed development, growth phase demarcation of the seed is imperative. Growth phases can be

determined with data of DMA and water content. The amount of dry matter represents seed capacity to accumulate photosynthates, and ultimately seed size. Seed enters into maturation phase when water content in the seed decreased. Thus the correlation between these two growth parameters helps in the determination of seed growth phases (Jhala and Thaker, 2015).

IAA and PAA levels in the developing seeds.

Figure 1a presents changes in IAA levels from anthesis until maturity. IAA levels were higher during early seed growth than in later stages; and increased during cell division and cell elongation phases (Fig. 1a) and peaked on the 5th day. It then declined and remained very low levels during the latter stages of seed development. Similarly, PAA levels were recorded higher in the early cell division stage, declined gradually in elongation phase and remained at very low levels during latter stages (Fig. 1b). When the ratio of PAA to IAA was worked out at given stage of growth and development of the seed, values were higher during early stages (data are not presented) than later growth phases. Overall PAA levels were generally slightly higher than that of IAA initially.

The role of auxins in cell division and elongation is elaborated in many systems (Whightman and Lightly, 1982; Patel and Thaker, 2004; Chudasama and Thaker, 2008). It was demonstrated that longer cotton fibers had more IAA and PAA than their shorter counterparts and addition of IAA or PAA to *in vitro* grown cotton fibers significantly increased the fiber length (Gokani and Thaker, 2002). Many plant systems were reported with high PAA than IAA at physiological concentrations (Whightman and Lightly, 1982). In our laboratory also in many systems like internodes development (Patel and Thaker, 2004), cotton fiber (Gokani and Thaker, 2002), cotton seed (Bhatt and Thaker, 2008) and seeds of *Cajanus cajan* (Chudasama and

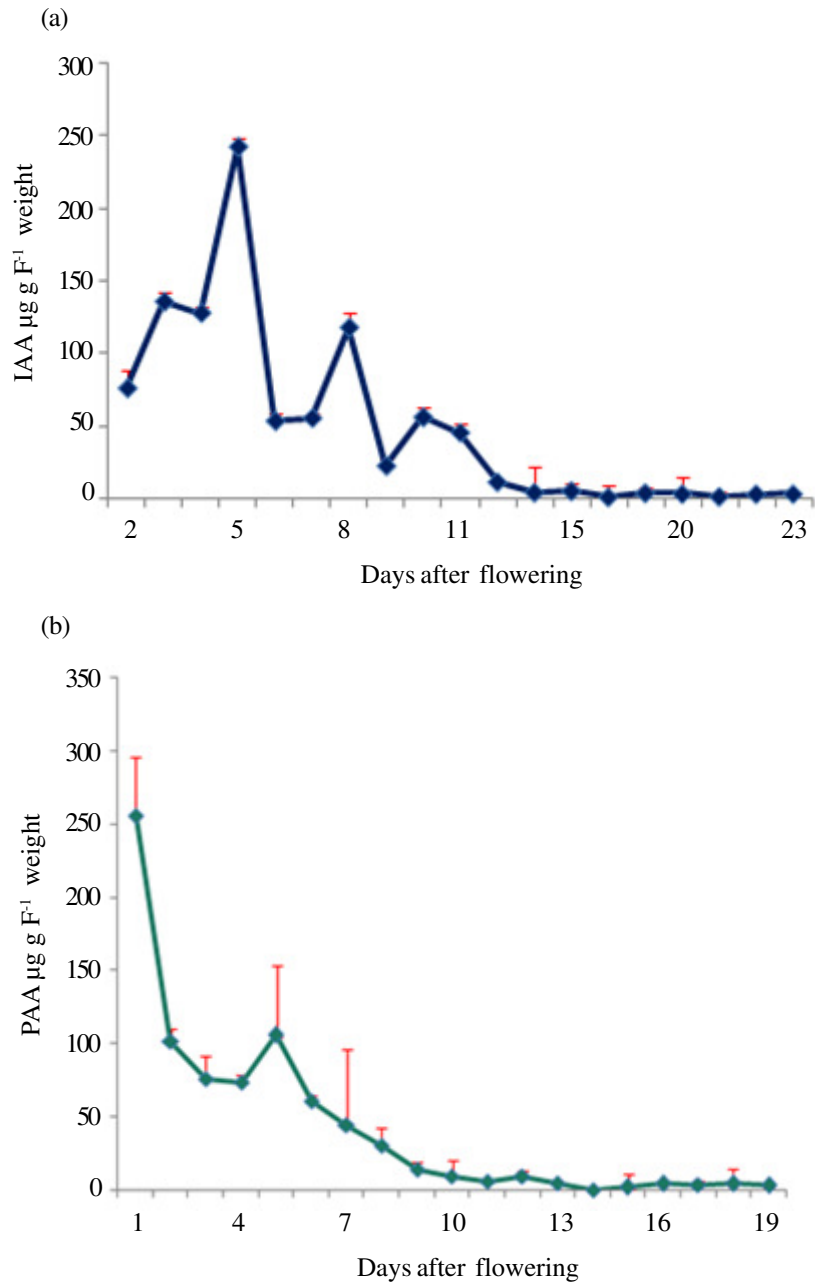


Figure 1. Change in IAA $\mu\text{g FW}^{-1}$ gm (a) and PAA $\mu\text{g FW}^{-1}$ gm (b); in variety GR-11 against seed age in days.

Thaker, 2008) higher PAA than IAA have been reported, suggesting that cells required more PAA than IAA for physiological functions. It is thus likely that IAA and PAA play important roles in cell division and elongation phases in rice seed as well; and hence sink size development.



ABP1 and TIR1 binding with auxins (IAA and PAA). Ligands used for docking are presented in Table 1. SWISS-MODEL (<http://swissmodel.expasy.org/>) was used for rice protein prediction. Properties of auxin binding protein, transport inhibitor response 1 (TIR1) and S-phase kinase-associated protein are presented in Figures 2a, b and c, respectively. Score of Predicted Protein (Table 2), in that Z-score, RMSD, Score, Align-length, Gaps, and Identity given for Predicted Protein of rice using Similarity Protein Structure Comparison Tool V 3.0.8 (jCE V.1.1). Docking results has been obtained for each ligand with the receptors was analysed with docking energy and interaction of each ligand (Table 3); rice ABP showed 90.00%, SKP1 showed 70.30% and TIR1 showed 76.45% similarity.

It was observed that PAA and IAA can dock with predicted proteins. IAA binds more efficiently with ABP1 (Table 3). PAA molecules are demonstrated for passive uptake of plant cells (Sibu and Petra, 2011); however, in the present study, PAA binds strongly to ABP1.

Similarly, docking of PAA with ABP1 from maize was also demonstrated (Lobler and Klambt, 1985) and PAA induced some conformation change in structure of ABP1 (Napier and Venis, 1990). This further supports the acclaim that the mechanism of PAA action may include ABP1. Although, the chemical nature of plant hormones does not predict the cellular location of the receptor, the binding of hormone to receptors caused a conformational change to initiate the transduction of the signal; and once activated, receptors could alter gene expression directly by acting as transcriptional factor (Skoog and Miller, 1957). The second class of auxin receptors is believed to be intracellular TIR1/AFB class receptors, in contrast with ABP1 as extracellular receptor (Xu *et al.*, 2014). Predicted proteins SKP and TIR1 of rice showed IAA binds more efficiently and TIR1 and SKP, together dock with IAA and PAA shows more efficiently with IAA.

Phylogenetic analysis and membrane prediction of *OsPINs*. PIN proteins act as auxin exporters, in rice *OsPIN1a*, *OsPIN1b*, *OsPIN1c*, *OsPIN1d*, *OsPIN2*, *OsPIN3a*, *OsPIN3b*, *OsPIN5a*, *OsPIN5b*, *OsPIN5c*, *OsPIN8*, and *OsPIN9* PIN protein sequences download from UniProtKB. Paponov *et al.* (2005) reported that Phylogenetic analysis using the detected *OsPIN* proteins and *AtPINs*

TABLE 1. Selected ligand list detail

Compound name	IAA	PAA
PubChem ID	802	999
Molecular Weight	175.18396 g mol ⁻¹	136.14792 g mol ⁻¹
Molecular Formula	C ₁₀ H ₉ NO ₂	C ₈ H ₈ O ₂
Canonical SMILES	C1=CC=C2C(=C1)C(=CN2)CC(=O)O	C1=CC=C(C=C1)CC(=O)O
3D Conformer		

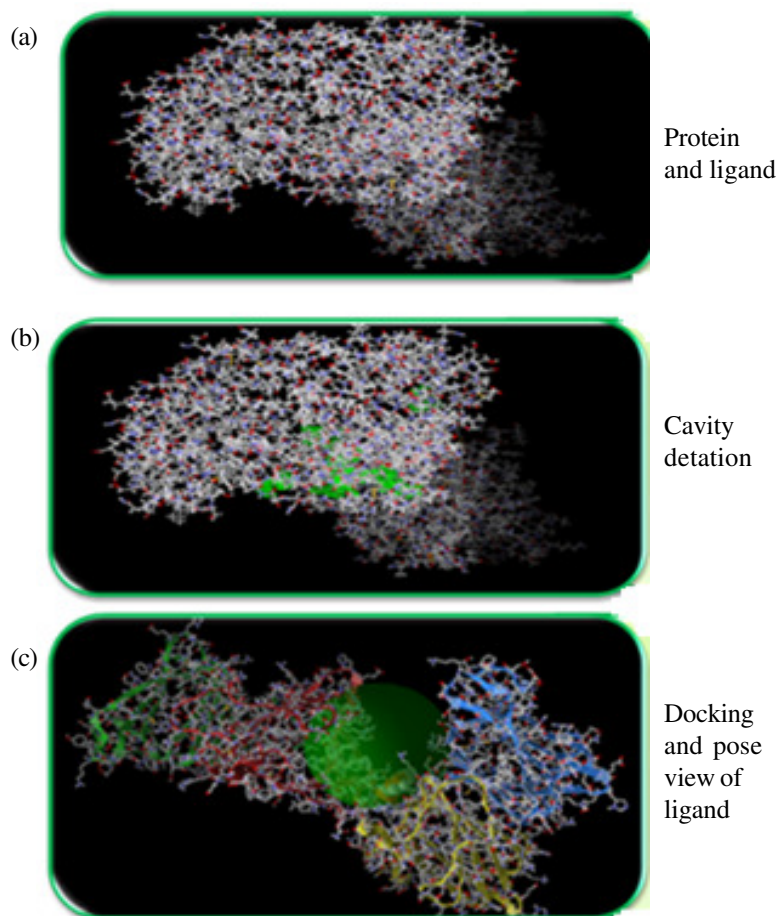


Figure 2 (a). Properties of Rice auxin binding protein (Swiss dock:<http://swissmodel.expasy.org/>), (b) Properties of Rice transport inhibitor response 1 (TIR1) (Swiss dock:<http://swissmodel.expasy.org/>), and (c) Properties of Rice S-phase kinase-associated protein (Swiss dock:<http://swissmodel.expasy.org/>)

TABLE 2. Score data of predicted protein in Protein Structure Comparison Tool V 3.0.8 (*jCE* V.1.1)

Protein name	Z-score	RMSD	Score	Align-length	Gaps	Identity	Similarity
RICEABP	7.93	0.43	840.48	320	0%	83.13%	90.00%
RICESKP1	5.33	1.06	190.76	165	24.50%	64.86%	70.30%
RICETIR1	8.4	0.59	1439.84	569	2.11%	60.81%	76.45%

showed similar results. The phylogenetic relationships between PINs of dicots and monocots were determined through a UPGAM phylogenetic analysis, which also allowed determination of the relationship of the PIN

auxin efflux carriers' family. Here we had used rice, *Zea mays* and *Arabidopsis* sequences for the same purpose. One clade PIN5 protein sequences of rice and maize is followed with *AtPIN5* sequence; while in the second clade,

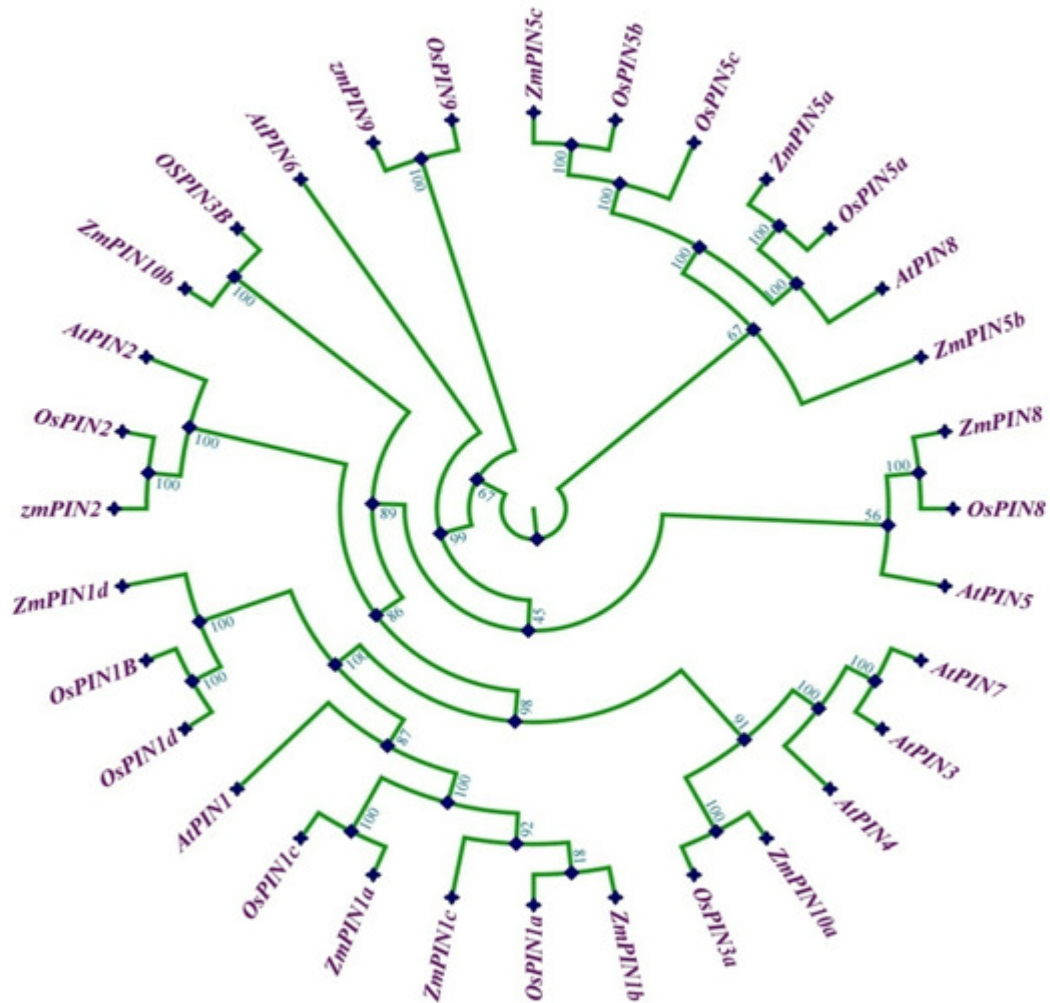


Figure 3. UPGMA Phylogenetic trees showing the relationship between *Athaliayana*(dicot), *Maize* and rice(monocot) PIN family proteins.

TABLE 4. List of the auxin transporters and putative transporters

Organisms	Name	Uniprot ID
<i>Oryza sativa</i>	OsPIN1a	Q0DXM9
	OsPIN1b	P0C0X5
	OsPIN1c	Q0DDD2
	OsPIN1d	D5A7I8
	OsPIN2	Q0DAE1
	OsPIN3a	D5A7J0
	OsPIN3b	Q6L5F6
	OsPIN5a	Q0JGK0
	OsPIN5b	D5A7J3
	OsPIN5c	Q0J484
	OsPIN8	Q0JJV0
	OsPIN9	Q0JIG6

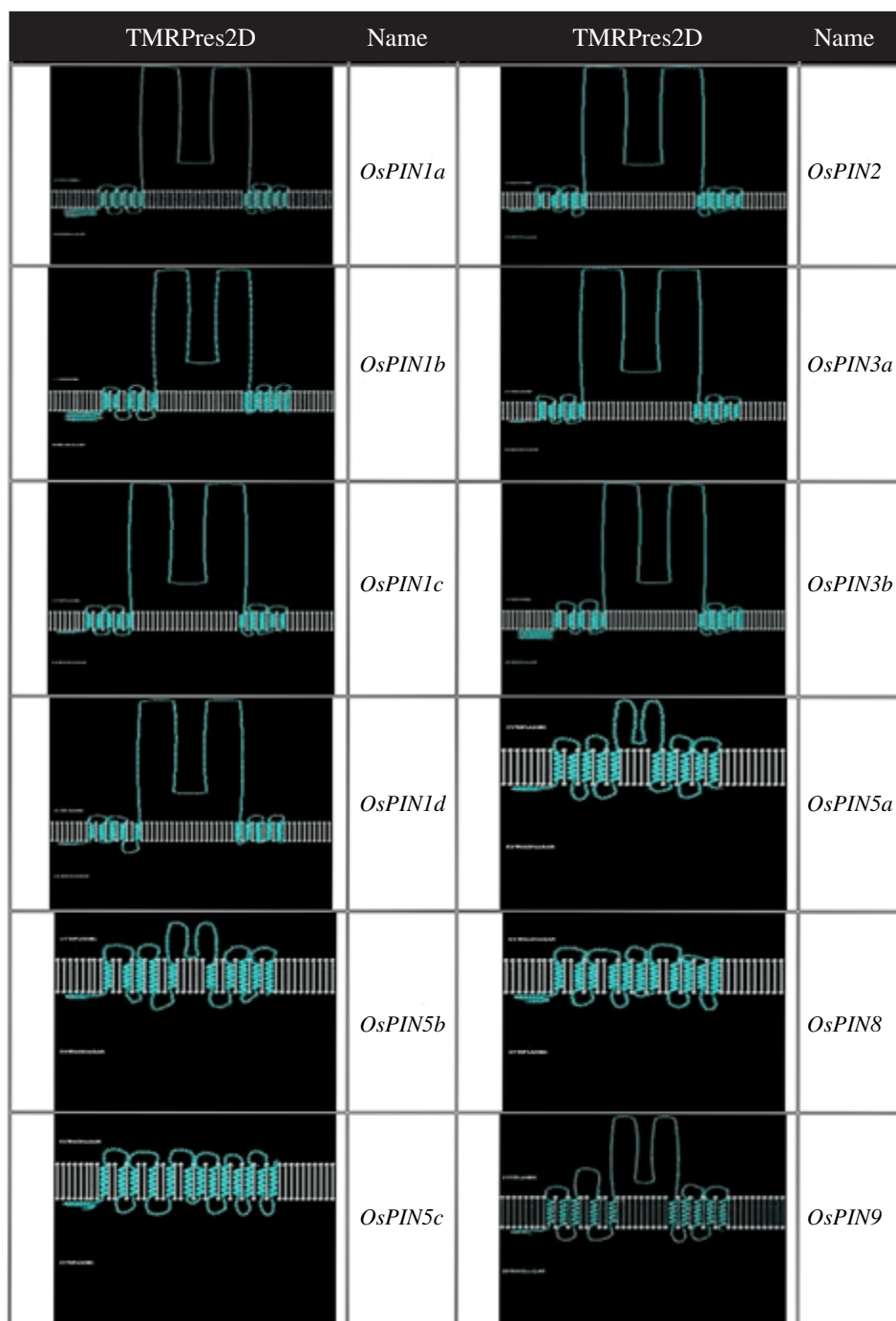


Figure 4. Predicted Membrane-spanning Helices of *rice* PIN Proteins PIN protein sequences were analysed by HMMTOP (<http://www.zimhu/hmmtop/>) Transmembrane prediction programmes.

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