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Research Article

Virtual Exploration and Annotation of EPAD1 gene in Poaceae

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ABSTRACT

Pollen grain is enclosed in the multi layered cell wall, combating chemical, physical and other stress conditions. The wall sculpturing of the pollen imparts substantial taxonomical evidence for the classification. This characteristic configuration is comprised by the accumulation of sporopollenin on the template primexine. Recently identified EPAD1 (Pollen Exine Pattern Designer) gene discovered to be responsible for the ornamentation of pollen wall. This research is aimed at the investigation of EPAD1 gene, confirmation of EPAD1 role in pollen wall sculpturing through virtual screening. EPAD1 gene homologs are determined in Poaceae, along with gene annotation, protein virtual modelling, conservation analysis, phylogenetic investigation and functional hits observation, manipulating BLAST, QuickGo, ChimeraX, Weblogo, MUSCLE and ExPasy-PROSITE respectively. Twenty-six homologs are investigated with highest identity 93% and query coverage 63% in *Oryza meyeriana*. EPAD1 annotation revealed that it performs in the pollen wall assembly, development, external encapsulating structure, gametophyte development, anatomical structures and morphogenesis. Orn/DAP/Arg decarboxylases family 2 structure 2 and twin arginine translocation (Tat) signals profile hits are observed on predicted homologs of Poaceae. The determined hits on EPAD1 functional analysis confirmed its inclusion in designing pollen morphology. The amino acids in the selected query coverage expressed high conservation for EPAD1 among grasses. Similarly, the phylogenetic evaluation represented the comparative relation and evolutionary patterns among the homolog species at genus level. Divergent species of the same genus *Triticum, Oryza, Panicum* and *Setaria* conjoint the corresponding note in phylogeny with the other species of similar genus. Briefly gene for pollen exine designing requisite to be investigated in other plant families, as it was discovered to be family specific. These outcomes will accelerate exploration of exine ornamentation and comparative plant families essay, based on the pollen exine designer gene.

Keywords: Pollen, exine, sporopollenin, ornamentation, gene, homology, ontology, Poaceae.

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INTRODUCTION

Pollen is the male gametophyte in flowering plants that is enclosed within a multilayer cell wall (Li et al. 2020). It directs the physical chemical resistance against the multiple environmental stresses (Shi et al. 2015). Pollen wall is a sculpture of inner intine and outer exine layers. Intine is regulated by haploid gametophyte

absolutely (pollen grain itself) and its prime constituent is cellulose. The exine is portioned into inner nexine (smooth) and outer sexine (variously sculptured). A comprehensive framework of the pollen walls clearly observes through the utilization of microscopic approach. The conserved structure of pollen wall is extensively recognized under a transmission electron microscope (TEM), (Bahadur et al. 2022a; 2022b). The distinctive arrangement of sexine is fabricated from a very resistant and complex polymer called sporopollenin (Quilichini et al. 2015). The process and the factors that regulate the assembly of the sporopollenin on the pollen wall surface, are under exploration (Li et al. 2020). A multitude of genes presumed to have an influence on the pollen walls patterning and coat structure like glycine-rich protein genes (GRPs in Brassicaceae) (Lu et al. 2020), merely a majority of them act on the composition and flow of components of the wall. *EPAD1* gene determination by Li et al. (2020) in rice, unveiled the species/family's specific disposition of sporopollenin on primexine. Sporopollenin composes the discrete organization by deposition on the template (primexine) and this is predicted to be regulated by *EPAD1* as by mutating the gene *EPAD1* the abnormal configuration developed, while the sporopollenin is produced normally (Li et al. 2020).

Genes that operate the formation of primexine and biosynthesis of sporopollenin are conserved and studied through the experiments on biochemical and comparative phylogenetics levels, among the land plants (Wallace et al. 2011; Shi et al. 2015). Orthologs of *EPAD1* are conserved in rice, sorghum, wheat (Li et al. 2020). Comparability in the protein sequence like the conserved domain of lipid transfer protein (LTP) containing eight-Cys and similar phenotypes after mutating the gene demonstrated the conserved nature of *EPAD1* in Poaceae (Kouidri et al. 2018), although variable C-terminal displayed divergence in species (Kouidri et al. 2018; Li et al. 2020), as aside from homogenous demonstration of ornamentation, slight variations prevail in sculpture and size between species (Mander & Punyasena 2016).

Genes studied in *Centrolepis aristata* (Centrolepidaceae) analog with *EPAD1* despite considerable variations. During evolution, the pollen ornamentation gene in plants may originate consequential to gene depletion (Shah et al. 2016). Homology-based hunting of genes possessing a percentage identity > 60, is stated significant (Cherian et al. 2022). In silico gene analysis is an excellent approach for the initial identification of key genes and gene clusters whose expression is altered at molecular levels (Rahman et al. 2022). In-silico investigations have been used for investigation of apoptosis mechanistic induction (Zaki et al. 2022), SARS-CoV-2 (Dwarka et al. 2020), animal and plant systems (Cherian et al. 2022; Dash and Ghag 2022; Rahman et al 2022) The discovery of nonspecific lipid transfer proteins (nsLTPs) in pollen exine ornamentation assisted the conception of the wall sculpturing procedure (Li et al. 2020). Investigation of *EPAD1* orthologs in members of Poaceae will further unfold the process of ornamentation on primexine. The role of *EPAD1* orthologs in other members of Poaceae and other families in pollen wall patterns is unexplored. In this study homologs of *EPAD1* are identified and characterized in the Poaceae family.

MATERIALS AND METHODS

Sequence Retrieval of EPAD1

EPAD1 gene sequence of *Oryza sativa Japonica* is fetched from NCBI (National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) with UniProtKB/Swiss-Prot ID: Q75GY0.1 and characterized as a full non-specific lipid transfer protein. Saved the amino-acids sequence in FASTA format.

Homolog Search

A Fetched sequence of *EPAD1* is BLAST against the available sequences of the Poaceae family manipulating BLASTp (Protein BLAST: search protein databases using a protein query (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and tBLASTn (tblastn: search translated nucleotide databases using a protein query. (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Homologs are saved in the FASTA format, with percentage identities, and query coverage.

Gene Ontology

EPAD1 gene is annotated via gene ontology online available bioinformatics database QuickGO (ebi.ac.uk). Retrieved the results for interpretation.

Virtual Modelling

Novel 3D protein structures are prepared/constructed for *EPAD1* gene in six species of grasses. The structures are generated using ChimeraX 1.3 practicing Alpha fold tool. The superlative models are retrieved and examined in the discovery studio visualizer (2021 Client). Constructed Hydrophobicity, Ramachandran, C-alpha, C-beta, and residue plots.

The Multiple Sequence Alignment (MSA) and Phylogenetic Tree Analyses

The MSA is analysed using Clustal Omega (MUSCLE < Multiple Sequence Alignment < EMBL-EBI). The phylogenetic tree is constructed using MEGA softare (https://www.ebi.ac.uk/Tools/msa/clustalo/). FASTA format of all sequences is submitted with default parameters. Neighbor-joining tree constructed, alignments, and cladogram of the phylogenetic tree are saved.

Conservation Analysis

Weblogo is a conservation analysis tool. It analyses the conservation of nucleotide/amino acid in specific regions among different sequences. The highly conserved regions (63 amino acids) of all the homologs of *EPAD1* in Poaceae are subjected to Weblogo (WebLogo - Create Sequence Logos (https://weblogo.berkeley.edu/logo.cgi). The Colour pattern is costumed for amino acids. Results are saved for further analysis.

Functional Hits/Domains identification

There are bioinformatics tools, that alongside variable query coverage, find the functional sites on the homologs sequences, confirming the sequence has an equivalent function with some evolutionary changes. ExPASy - PROSITE tool (https://prosite.expasy.org/) hunts the sites/hits on the protein and DNA sequences. Sequences are submitted in FASTA format. Saved the results of the observed hits.

RESULTS

The homologs search of EPAD1 gene in Poaceae plants available data sets at nr and gss displayed the promising results. Maximum query coverage 63% is observed for *Oryza meyeriana* and *Oryza brachyantha.* The range of query coverage in the homologs of EPAD1 is from 23% to 63%. Grasses with less than 23% query coverage are not selected in this study. The percentage identity for the covered query varied from 42.59% to 93.83% (Table 1). Only two grasses exhibit the percentage identity below 50%. Highest percentage identity 96.88 % is noticed in *Zizania palustris.*

Gene ontology establish all of the gene's characteristics, including whether it is a part of a process, function in a process, or component of a process. The annotation of the EPAD1 gene confirm its direct function in the pollen wall assembly (Black colour first direct line for function Fig 1) which is a part of pollen development process in the second step (Blue colour line Figure 1). This gene function in cellular component assembly, in external encapsulating structure, gametophyte development, anatomical structure formation. The biological functions of this gene are categorized into cellular process, developmental process and multicellular organismal process. The developmental process includes anatomical structure morphogenesis and development, gametophyte development, cellular development, pollen development. Cellular processes comprise of cellular organization, biogenesis, morphogenesis, assembly, pollen wall assembly.

The 3D protein structured are constructed virtually for EPAD1 homologs in *O. brachyantha, O. meyeriana, O. sativa, O. sativa, T. aestivum* and *Z. mays* (Fig 6). The number of amino acids is 233, 234, 166, 169, 214 and 240 in *O. brachyantha, O. meyeriana, O. sativa, O. sativa, T. aestivum* and *Z. mays* respectively. The folding and stability of the constructed structures are determined via Ramachandran plots (Figure 8). Ramachandran plots results for EPAD1 homologs in *O. brachyantha, O. meyeriana, O. sativa, O. sativa, T. aestivum* and *Z. mays* revealed that in favoured region maximum number of amino acids are present while only few falls in not allowed area representing them as good model for molecular stability and specific interactions. The hydrophobicity plots for the designed structures of EPAD1 homologs revealed that all these grasses possessed considerable number of hydrophobic amino acids (Fig 7).

The homologs of EPAD1 are subjected to hierarchical cluster analysis (UPGMA). The outcomes of the cluster analysis are presented in Figure 4. There is one major and two small clusters. *Setaria italica* and *Setaria viridis* are in the single smallest cluster, closest to each other. The second small cluster consist of four species, three species of genus *Panicum* are in this same cluster. Major cluster delineated into number of variable subclusters. Members of *Oryza* are in the similar cluster, sharing the closest note. *Triticum urartu* and *Triticum turgidum* are also phylogenetically closed based on the EPAD1 gene sequence.

Within the gene sequence, the conservation of amino acids is analysed among the predicted EPAD1 gene sequences of selected grasses. The selected region of 63 amino acids for each plant expressed high conservation among the studied taxa. The colour in different alphabets represent the different amino acids and the length of the alphabets show its conservation status in the studied Poaceous members (Figure 5). Two types of functional hits or domains on the homologs of EPAD1 gene are detected. Twenty plants possessed Orn/DAP/Arg decarboxylases family 2 structure 2 functional hit. Whereas *Eragrostis curvula* is observed with twin arginine translocation (Tat) signals profile (Figure 2).

No hits are observed on *Oryza sativa Indica, Hordeum vulgare, Aegilops tauschii, Triticum turgidum* and *Triticum Urartu.*

Table 1. EPDA1 homologs in Poaceae family (nr = non-redundant protein sequences, gss = genomic survey sequence)

S.No	Plant Name	Database	Sequence ID	Percentage Identity %	Query Coverage %	Number of amino acids
1	*Oryza meyeriana*	nr (BLASTp)	KAF0910051.1	93.83	63	233
2	*Oryza brachyantha*	nr (BLASTp)	XP_040378633.1	82.67	63	234
3	*Oryza officinalis*	gss (tBLASTn)	DU542029.1	67.65	44	166
4	*Oryza barthii*	gss (tBLASTn)	KS499818.1	67.65	44	169
5	*Oryza punctata*	gss (tBLASTn)	CW529515.1	67.65	44	214
6	*Oryza minuta*	gss (tBLASTn)	CZ678605.1	65.69	44	240
7	*Oryza sativa indica*	nr (BLASTp)	EEC75870.1	69.29	37	236
8	*Zizania palustris*	nr (BLASTp)	KAG8062008.1	96.88	28	237
9	*Digitaria exilis*	nr (BLASTp)	CAB3487128.1	79.69	28	114
10	*Panicum hallii*	nr (BLASTp)	PUZ47267.1	81.25	28	214
11	*Panicum virgatum*	nr (BLASTp)	XP_039771960.1	79.69	28	243
12	*Panicum miliaceum*	nr (BLASTp)	RLM74730.1	79.69	28	215
13	*Dichanthelium oligosanthes*	nr (BLASTp)	OEL14774.1	79.69	28	210
14	*Setaria italica*	nr (BLASTp)	XP_004978021.1	79.69	28	215
15	*Setaria viridis*	nr (BLASTp)	XP_034605203.1	79.69	28	216
16	*Triticum aestivum*	nr (BLASTp)	QBL97888.1	88.89	27	98
17	*Brachypodium distachyon*	nr (BLASTp)	XP_003559610.1	92.06	27	228
18	*Triticum dicoccoides*	nr (BLASTp)	XP_037424533.1	88.89	27	216
19	*Hordeum vulgare*	nr (BLASTp)	XP_044972473.1	76.19	27	330
20	*Sorghum bicolor*	nr (BLASTp)	XP_002447893.1	76.19	27	218
21	*Zea mays*	nr (BLASTp)	NP_001140747.1	77.78	27	217
22	*Miscanthus lutarioriparius*	nr (BLASTp)	CAD6260803.1	73.02	27	219
23	*Aegilops tauschii*	nr (BLASTp)	XP_020183022.1	68.25	27	410
24	*Eragrostis curvula*	nr (BLASTp)	TVU04238.1	55.56	27	210
25	*Triticum turgidum*	nr (BLASTp)	VAH09308.1	40.91	27	214
26	*Triticum Urartu*	nr (BLASTp)	EMS51262.1	42.59	23	328

DISCUSSION

Li et al. (2020) discovered the exine pattern designing gene EPAD1 in *Oryza sativa* Japonica and determined it as a grass specific gene. In this study EPAD1 homologs are investigated in Poaceae family available sequences at BASTp and tBASTn. Non-redundant protein sequences (nr) and genomic survey sequence (gss) yielded acceptable homologs of this gene in Poaceous taxa. Twenty-six species of the Poaceae family revealed homology with EPAD1 gene, exhibit considerable percentage identities and query coverage (Table 1). Homology search present the foundation for the discovery of unidentified genes in the online available sequences. Percentage identity and query coverage in the studied grasses ranged from 43% to 94% and 23% to 63% respectively. Query coverage of *Oryza meyeriana, Oryza brachyantha, Oryza officinalis, Oryza barthii, Oryza punctata* and *Oryza minuta* ranged from 44-63%.

Gene ontology display the biological function of gene by integrating the illustration of the gene and their products from all species. Regulation of the biological mechanisms at each functional level can be depicted via gene ontology (Conesa et al. 2005). Ontology of the EPAD1 gene disclosed its functions in pollen exine sculpturing i.e being a part of the processes like external encapsulating structure, pollen wall assembly, pollen development, gametophyte development, anatomical structures (Fig 1). Cherian et al. (2022) analyse ontology of tRF revealing that biological

mechanisms like reproduction, stress responses, growth and photosynthesis control through various genes regulated via tRF.

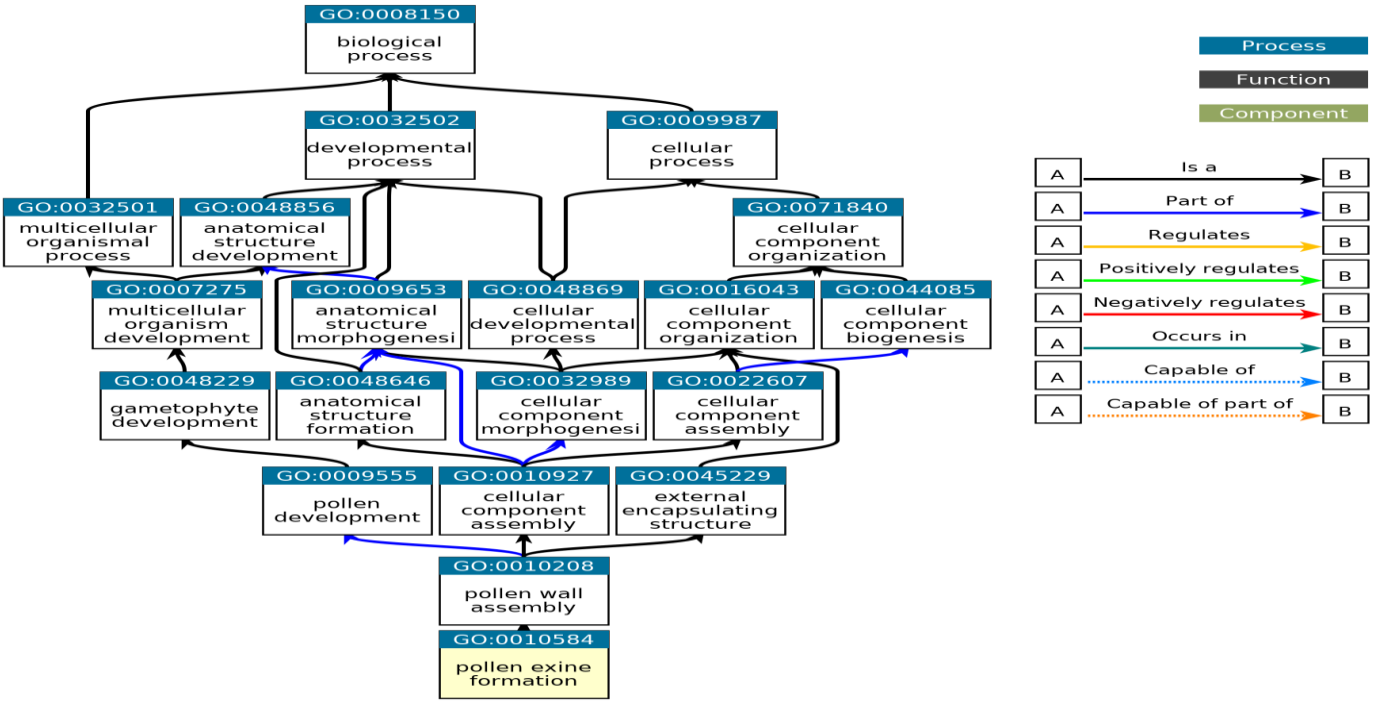

Figure 1. Ontology of EPAD1 gene.

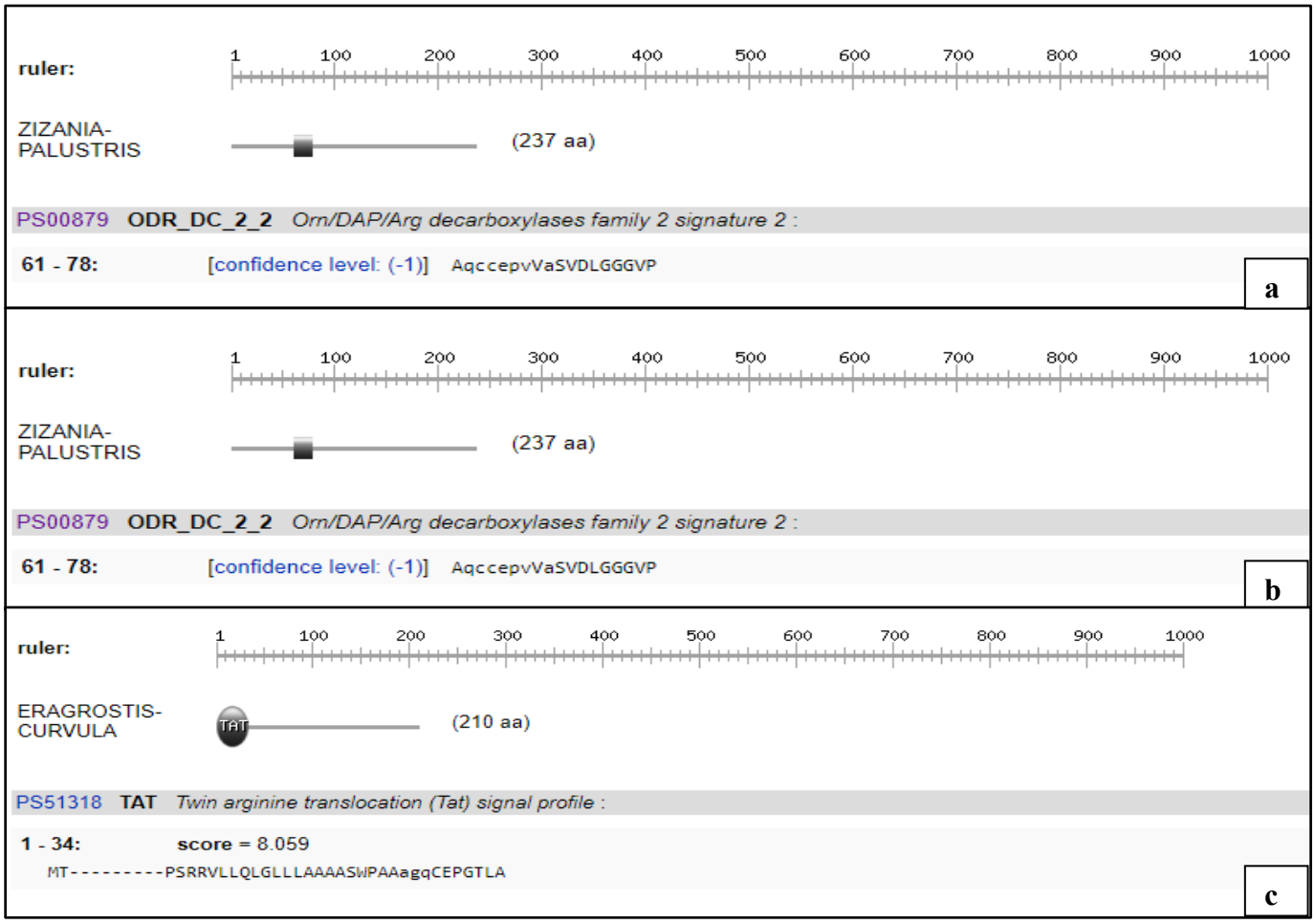

Figure 2. Hits on EPDA1 gene and its homologs in Poaceae (a, b, c).

Conserved hits/domains identification and wet lab verification validate the predicted homologs (Prakash et al. 2010). In this research functional hits on EPAD1 and its homologs are examined through PROSITE (Bairoch & Bucher 1994). PROSITE provides characterization of proteins, their functions as it is a compilation of patterns and sites of protein. It is operated under the EMBL (European Molecular Biology Laboratory). Orn/DAP/Arg decarboxylases family 2 structure 2 and twin arginine translocation (Tat) signals profile were the detected hits on the 21 Poaceous members (Fig 2).

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Triticum-urartu                FVAAHVLLSCDPLLP---TAGCCN----ALLGSVPRDDALPCLCAAAHDPDLQRA-GYME
Triticum-turgidum              QLATAIVKNCVEDFEPTNAAACCS----SVLPTVDMAG---CLCLVVDEPQLALS-GT--
Eragrostis-curvula             TLAPGVALCCTFRLP---TAICCR----ALVHSVTVGGGTGCLCRLAAERVVINT-AL--
Panicum-hallii                 NLATQITLFCMPDMP---TAPCCE----PVVASVDLGGGVPCLCRVAAQPQLVLA-RL--
Dichanthelium-oligosanthes     NLATQITLFCMPDMP---TAPCCE----PVVASVDLGGGVPCLCRVAAQPQLVLA-RL--
Panicum-virgatum               NLATQITLFCMPDMP---TAPCCE----PVVASVDLGGGVPCLCRVAAQPQLVLA-RL--
Panicum-miliaceum              NLATQITLFCMPDMP---TAPCCE----PVVASVDLGGGVPCLCRVAAQPQLVLA-RL--
Setaria-italica                NLATQITLFCMPDMP---TAPCCE----PVVASVDLGGGVPCLCRVAAQPQLVLA-RL--
Setaria-viridis                NLATQITLFCMPDMP---TAPCCE----PVVASVDLGGGVPCLCRVAAQPQLVLA-RL--
Digitaria-exilis               NLATQITLFCMPDMP---TAPCCE----PVVASVDLGGGVPCLCRVAAQPQLVLA-RL--
Zea-mays                       IIATQIALFCMPDMP---TAPCCE----PIIASVDLGGGVPCLCRVAAQPQLVLA-RL--
Sorghum-bicolor                IIATQIALFCMPDMP---TAPCCE----PIIASVDLGGGIPCLCRVAAQPQLVLA-RL--
Miscanthus-lutarioriparius     IIATQIALFCMPDMP---TAPCCE----PIIASVDLGGGIPCLCRVAAQPQLVLA-RL--
Oryza-brachyantha              VLATQVSLFCAPDMP---TAQCCE----PVVASVDLGGGVPCLCRVAAEPQLIIS-GL--
Oryza-sativa-Indica            ILATQVSLFCAPDMH---TPRSAASRWWPPSTS---GGGVPCLCRVAARAAASSSPAS--
Oryza-meyeriana                ILATQVSLFCAPDMP---TAQCCE----PVVASVDLGGGVPCLCRVAAEPQLIIS-GL--
Oryza-sativa-Japonica          ILATQVSLFCAPDMP---TAQCCE----PVVASVDLGGGVPCLCRVAAEPQLIIS-GL--
Zizania-palustris              ILATQVSLFCAPDMP---TAQCCE----PVVASVDLGGGVPCLCRVAAEPQLIVS-GL--
Hordeum-vulgare                LLATQAALFCAPDMP---TTQCCE----PVIAAVDLGGGVPCLCRVAAEPHLVMD-GL--
Aegilops-tauschii              LLAPQVALFCAPDFS---T-QCCE----PVVPAINLSGCVPCVCRVPAEPQLVMA-GL--
Brachypodium-distachyon        LLATQVSLFCAPDMP---TAQCCE----PVVASVDLGGGVPCLCRVAAEPQLVMA-GL--
Triticum-aestivum              LLATQVALFCAPDMP---TAQCCE----PVVAAVDLGGGVPCLCRVAAEPQLVMA-GL--
Triticum-dicoccoides           LLATQVALFCAPDMP---TAQCCE----PVVAAVDLGGGVPCLCRVAAEPQLVMA-GL--
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Figure 3. The multiple sequence alignment (MSA) analysis of EPDA1 homologs in Poaceae members.

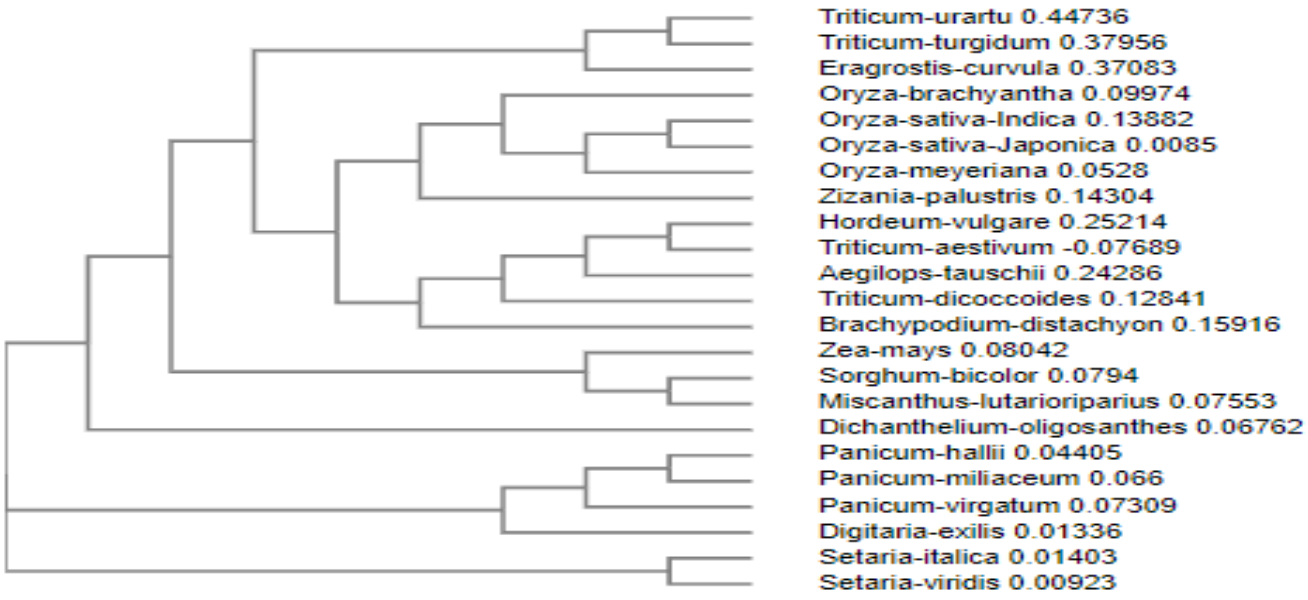

Figure 4. Phylogenetic relationship of EPDA1 gene of *Oryza sativa* Japonica and its homologs in Poaceous taxa.

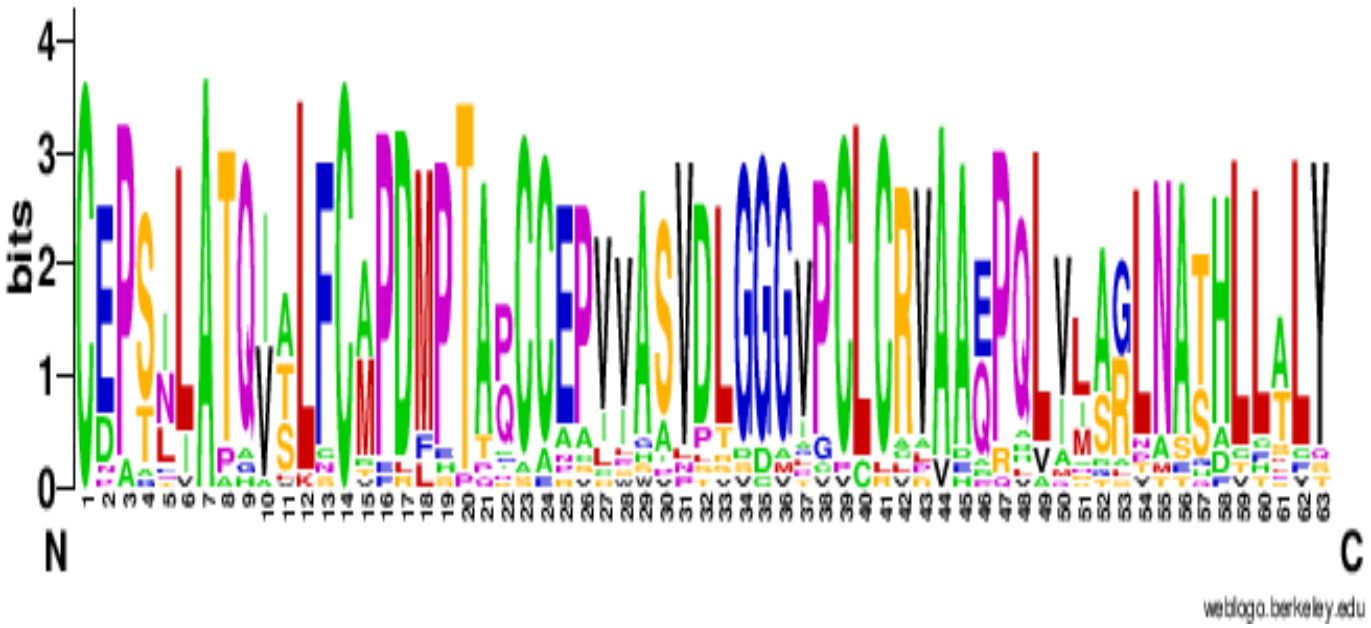

Figure 5. Conservation analysis of EPDA1 and its homologs in Poaceae members.

These hits observed to be associated in the pollen formation and development. Mitochondrial Tat (B) involved in the pollen formation along with the embryo development and vegetative growth (Schafer et al. 2020). An intron in 5' UTR of arginine decarboxylase family codes for the pollen specific expression (Peremarti et al. 2010). *Oryza sativa Indica, Hordeum vulgare, Aegilops tauschii, Triticum turgidum* and *Triticum Urartu* are not detected with these hits.
Virtual modelling for the EPDA1 is carried using ChimeraX and then visualized them in the discovery studio visualizer. ChimeraX is a next generation software, that is employed for virtual modelling to construct the 3D structures, their visualization, density mapping, medical imaging data with the ease of access and free download version (Goddard et al. 2018; Pettersen et al. 2021). Hydrophobic regions are highlighted in constructed proteins of EPAD1 (Fig 6,7).

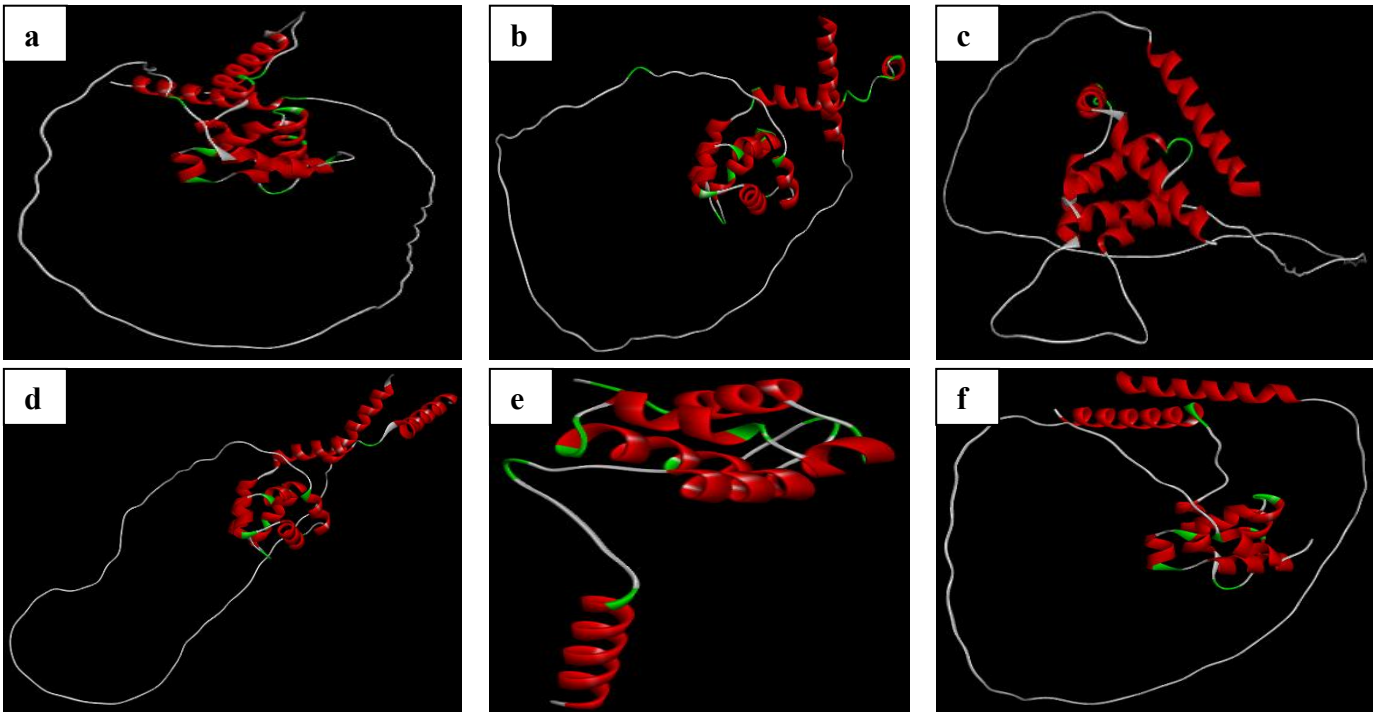

Figure 6. EPDA1 protein structures of O. brachyantha, O. meyeriana, O. sativa I, O. sativa J, T. aestivum, Z. mays a, b, c, d, e, f respectively.

The hydrophobicity plots of EPAD1 proteins helped to evaluate the protein structures as water mediates the protein-protein interactions. This provides large area protein-protein interfaces (temporarily stable and close), by compacting the individual proteins (Bhadra 2020). C-alpha, C-beta, residue chart, Ramachandran and hydrophobicity plots are constructed for EPDA1 in six species of grasses homologs of EPAD1 (Fig 8). C-alpha is constructed for central chain amino acids (Dhorajiwala et al., 2019). Using torsion angles, Ramachandran plots provide details regarding the description of polypeptide and confirmation of protein, as it is prime concept in structural biology. It is the reason of their widespread use.

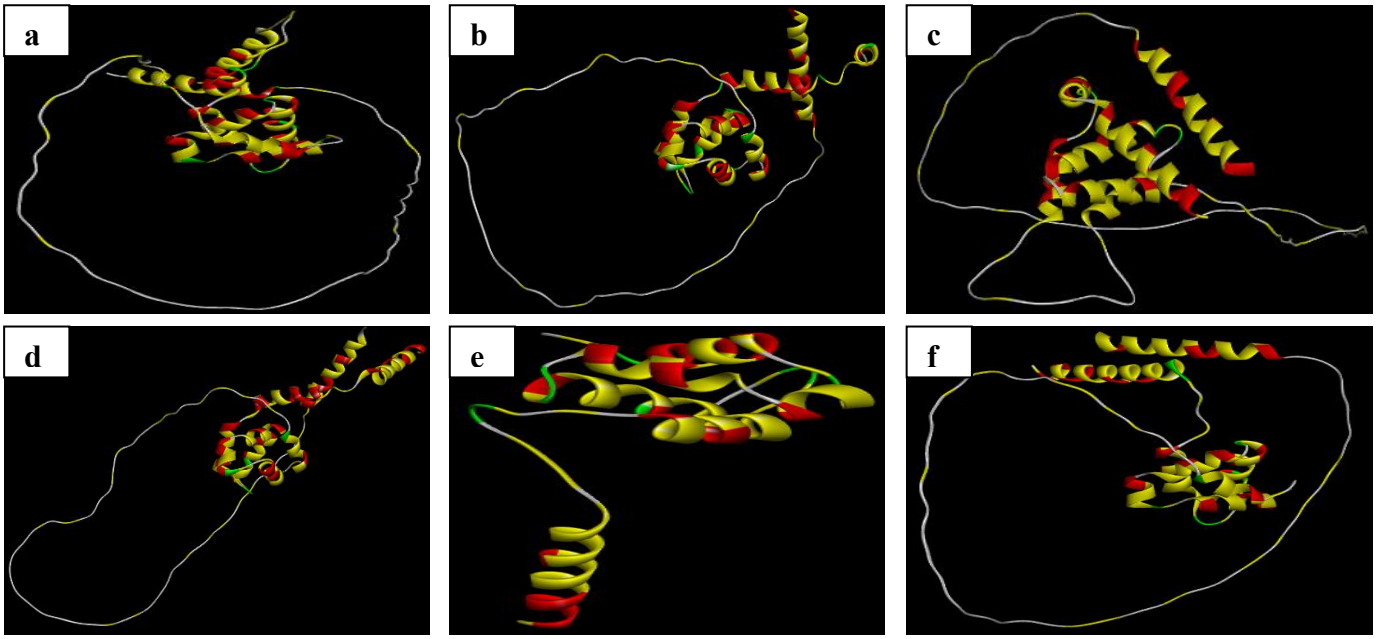

Figure 7. EPDA1 protein structures with highlighted hydrophobic amino acids of O. brachyantha, O. meyeriana, O. sativa I, O. sativa J, T. aestivum, Z. mays a, b, c, d, e, f respectively

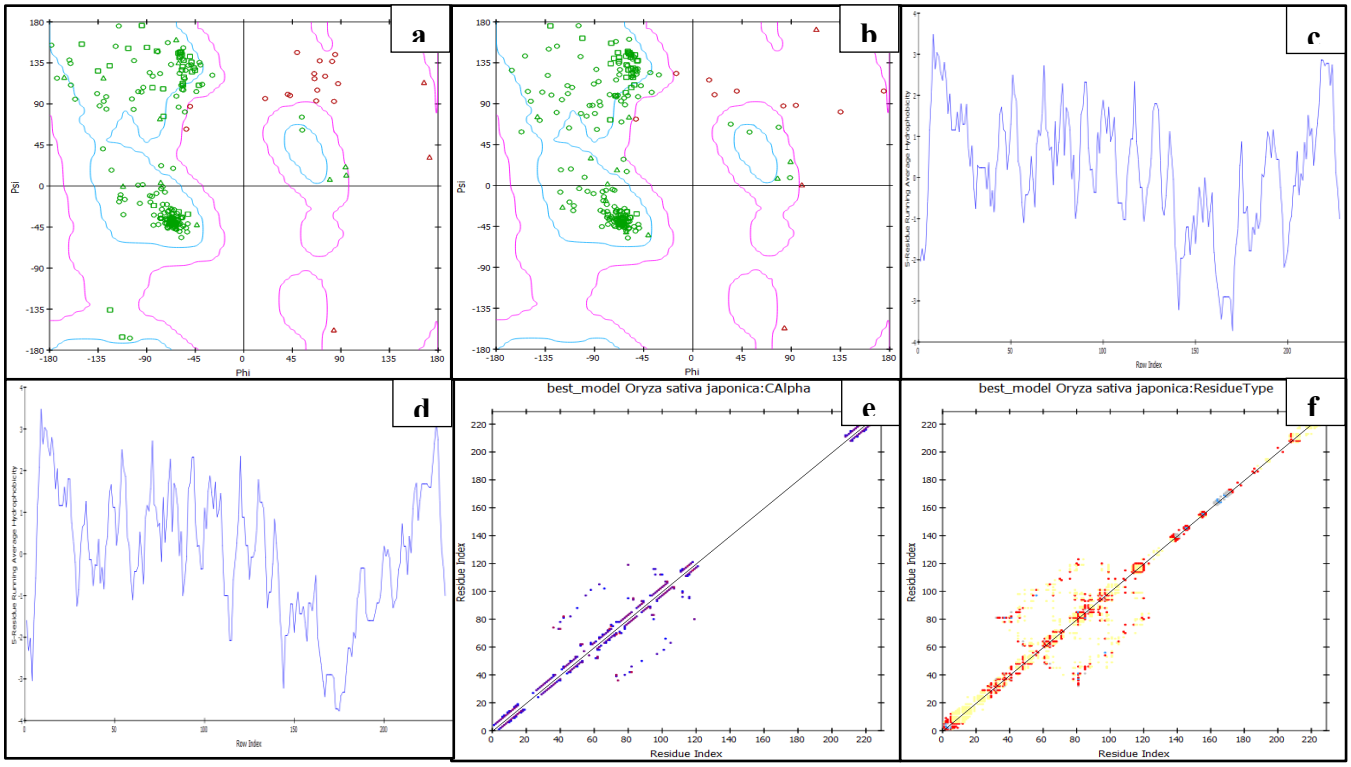

Figure 8. Ramachandran and hydrophobicity plots for EPDA1 proteins of *O. sativa J* and *O. brachyantha a, b, c, d* respectively. The C-alpha and residue plots for *O. sativa J* e and f respectively.

Ramachandran also named as ϕ, ψ-plot, became necessary segment for protein structure (Bhadra 2020). These plots provide understanding of backbone dihedral angles, that are allowed in energetically allowed regions, providing details about how the protein will fold and its stability (Ramachandran et al. 1963). Likewise, to evaluate the protein structure, hydrophobicity plots were constructed, as protein interactions are mediated by water (Bhadra 2020). EPAD1 structural analysis revealed the presence of majority of hydrophobic amino acids in its sequence (Fig 7). Similarly, the invitro assay of EPAD1 (Li et al. 2020) investigated its binding to phospholipids. It was proposed that in the regulatory protein arrangement and recruiting on the primexine to create the exact ornamentation, is caused by the involvement of the bound plasma membrane lipids to EPAD1.

For the determination of evolutionary relationship among the studied plants, multiple sequence alignment is performed using program MUSCLE (Edgar 2004) (Prakash et al. 2010). Phylogenetic analysis based on EPAD1 gene unveil evolutionary patterns in EPDA1 among grasses. Neighbour joining tree construction is a novel way that utilize molecular data for the construction of phylogenetic relationship, ultimately uncover the operational taxonomic units (OTUs). In comparison to the five procedures that use unweighted pair technique, probing OTUs is a precise approach (Saitou and Nei 1987). Species of *Oryza, Panicum, Setaria* and *Triticum* shared the same notes with species of the same genus (Figure 3,4). Phylogenetic relationship expressed the evolutionary conserved nature of the gene at genus level (Hu et al. 2008) in *Oryza, Panicum, Setaria* and *Triticum*. Regions with high query coverage provide the observation of the gene origin and its conservation (Prakash et al. 2010). Conservation analysis disclosed the existence of conserved amino acids in EPAD1 gene homologs among grass species, concluding the similar origin of EPAD1 gene grasses (Figure 5).

CONCLUSIONS

Conclusively the homologs of newly discovered EPAD1 gene are searched in Poaceae using Insilco approach, utilizing NCBI, BLAST, QuickGo, MUSCLE, Weblogo, PROSITE tools. These genes can be validated through wet lab in future research. Moreover, other plant families required to be explored for their specific exine pattern designer gene. The outcomes are helpful in new genes identification, determining the evolutionary patterns, gene annotation, conservation status and functional hits identification.

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Not applicable.

AUTHOR CONTRIBUTIONS

All the authors contributed equally to this research.

COMPETING OF INTEREST

No conflicts of interest have been disclosed by the authors.

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