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Physicochemical Analysis and Homology Modeling of Antioxidant Proteins of Foxtail Millet (*Setaria Italica*)

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Abstract

Foxtail Millet (*Setaria italica*) though falls in the category of neglected underutilized species (NUS), is considered to have very high nutritive values. It contain components that have high antioxidant activities and can reduce the blood sugar level. In this study, a bioinformatics and molecular modeling approach was adopted to explore properties and structure of foxtail millet antioxidant proteins. These antioxidant proteins include peroxiredoxins (PRDX), cysteine peroxiredoxin (CYS-PRX), ascorbic peroxidase (APX), dehydro ascorbate reductase (DHAR), and 2-Cysperoxiredoxin BASI (2CPs). Physico-chemical characterization interprets properties such as isoelectric point (pI), extinction coefficient (EC), aliphatic index (AI), instability index (II) and GRAVY and provides data about these antioxidant proteins and their properties. Prediction of motifs, patterns, disulfide bridges and secondary structure were performed for functional characterization. Three dimensional structures for these proteins were not available as yet at PDB. Therefore, homology models for these antioxidant proteins were developed. The modeling of the three dimensional structure of these proteins shows that models generated by Swiss Model were more acceptable in comparison to that by Geno 3D Model. The models were validated using protein structure checking tools PROCHECK and WHAT IF. These structures will provide a good foundation for functional analysis of experimentally derived crystal structures.

Keywords

Foxtail millet (*Setaria italica*); Antioxidant proteins; PRDX; CYS-PRX; APX; DHAR; 2CPs; *Insilico* analysis; 3D Homology modelling

Introduction

Foxtail millet (*Setaria italica*), a second highest growing small millet, is highly nutritious, non-glutinous, considered to be one of the least allergenic and most digestible grains available [1,2]. It contains a myriad of beneficial nutrients. Besides nutrients, grains have an abundance of phytochemicals, particularly phenolic compounds. Phytochemicals are biologically active organic substances of plant origin having disease preventing and health promoting properties. Most of the phytochemicals like polyphenols, flavonoids are found to have oxidant activity [3].

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The classical source of enzymatic and nonenzymatic antioxidants is plant- derived polyphenols obtained from a wide array of plant-based foods and medicinal plants. The search for natural antioxidants is an ongoing endeavour as an aid to combat the harmful effects of free radicals. In recent years some plant hydrolysates including foxtail millet have been shown to exhibit potent antioxidant properties [4,5].

It has been observed that the biological function of antioxidant proteins is based on the amino acid composition and sequence. In fact, the levels, compositions and properties of free amino acids and peptides have been correlated to the antioxidant activities of protein hydrolysates [6]. Certain structural characteristics such as the nature, charge, basicity, aromaticity and hydrophobicity of key amino acids in the primary sequence influence the three dimensional (3D) conformation of a peptide and therefore affect the biological function [7,8]. A strong structure- function relationship can be used to explain the antioxidative properties of most proteins. Experimental methods used to characterize a protein involve high cost and time frame. Further, determination of protein structure through X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy is very costly and time consuming. The *in silico* approaches provide a viable solution to this problem. Computational tools provide researchers to understand physicochemical and structural properties of the proteins. Since the widely available digital information about foxtail millet plant genome and its products has triggered the use of *in silico* identification of important proteins in this crop. However, a detailed analysis of the antioxidant protein sequences of foxtail millet, their probable structure, physicochemical and functional characterization has yet to be accomplished. In view of the above facts, the *in silico* identification, analysis and homology modeling of antioxidant proteins in foxtail millet has been carried out in present study.

Materials and Method

Sequence retrieval

The FASTA sequence of Foxtail millet antioxidant proteins (Table 1) were retrieved from NCBI's Protein database [9].

Physico-chemical characterization

For physico-chemical characterization theoretical isoelectric point (pI), molecular weight, total number of positive and negative residues, extinction coefficient, instability index, aliphatic index and grand average hydropathy (GRAVY) were computed using Expasy's ProtParam server [10].

Functional characterization

The SOSUI signal server was used for identification of transmembrane regions of antioxidant proteins. Since disulphide bonds are very essential in determining the functional linkages and stability of a particular protein, their presence and their bonding pairs were predicted by the tool CYS_REC. Information regarding antioxidant protein families, domains and functional sites was computed through Prosite server.

Secondary structure prediction

SOPMA (Self Optimized Prediction Method with Alignment)

Table 1: Antioxidant Proteins of Foxtail Millet considered for the study.

Antioxidant proteins	Accession no.	Sequence Length	Description
PRDX	XP_004969519.1	162	Predicted:peroxiredoxin-2C-like
	XP_004951605.1	227	Predicted:peroxiredoxin-2E-2, chloroplastic like
CYS PRX	XP_004958464.1	221	Predicted: 1-CYS peroxiredoxin PER 1-like
APX	XP_004958804.1	250	Predicted: L-Ascorbate peroxidase 2, cystosolic like isoform X2
DHAR	XP_004965063.1	274	Predicted: Glutathione S-transferase DHAR 3,chloroplastic like isoform X1
2-CPs	XP_004952670.1	260	2-CYS peroxiredoxin BAS 1,chloroplastic like

was employed for the secondary structural features prediction, properties and evolutionary history. The secondary structure were predicted by using default parameters (Window width: 17, similarity threshold: 8 and number of states: 4).

Model Building, evaluation and visualization

The 3D structure of antioxidant proteins under study is not yet available at Protein Data Bank. So the modeling of the three dimensional structure of the protein was performed by two homology modeling programs, Geno 3D [11] and SwissModel [12]. The evaluation and validation of the models was done by PROCHECK and WHAT IF softwares. The final modeled structures were visualized by PyMol and Swiss Pdb viewer.

Result and Disscussion

The amino acid composition of sequences of six antioxidant proteins, detected in foxtail millet genome was computed using Expasy PortParam tool from the primary structure analysis results and tabulated in Table 2. All the studied antioxidant proteins were found rich in aliphatic amino acids especially Ala, Asp, Gly, Leu, Lys and Val. Net percentage of hydrophobic amino acids was higher in all the antioxidant proteins except CYS-PRX. Higher percentage of hydrophobic amino acids increases solubility of proteins in lipid phase. Therefore, they are able to inhibit lipid peroxidation at the water-lipid interphase [13].

Physico-Chemical Characterization

Parameters computed using Expasy's ProtParam tool are presented in Table 3. The total number of amino acid residues in studied foxtail antioxidant proteins ranged from 162 to 274 with variable molecular weights. The computed Isoelectric point (pI) value of PRDX (XP_004969519.1), CYS-PRX, APX, DHAR and -CPs were less than 7 (pI<7) indicates that these antioxidant proteins were acidic. The pI of PRDX (XP_004951605.1) was greater than 7 (pI>7) revealed the basic nature of this protein. Isoelectric point is the pH at which the surface of protein is covered with charge but net charge of the protein is zero. Hence, the solubility of the protein is least and mobility in electrofocusing system is zero. In other words we can say at pI proteins are stable and compact. Thus, the computed isoelectric point will be usefull for developing buffer system for purification by isoelectric focusing method. Extinction coefficient (EC) of antioxidant proteins at 280 nm was ranging from 8542.5 to 27117.5 $M^{-1}cm^{-1}$ with respect to the concentration of Cys, Trp and Tyr. The high EC value of DHAR indicated the presence of high concentration of Cys, Trp and Tyr amino acids components. The computed EC value helps in the quantitative study of protein-protein and protein- ligand interactions in the solution. The value of instability index (II) provides an estimate of the stability of protein in a test tube. A protein whose II value is smaller than 40 (II<40) is predicted as stable, value above 40 predicts that the protein may be unstable [14]. The instability index (II) value for the foxtail millet antioxidant proteins were found to be ranging from 33.77 to 43.65. The result classified CYS-PRX, APX, DHAR and 2-Cps as stable protein and PRDX (XP_004969519.1 and XP_004951605.1) as unstable protein. The aliphatic index (AI), which is defined as the relative volume of a protein occupied by aliphatic side chains (A,V,I and L),of antioxidant proteins of foxtail millets ranged from 73.32 (APX) to 99.38 (PRDX -XP_004969519.1) among the retrieved sequences. It indicates that these antioxidant proteins may be stable for a wide temperature range. Low GRAVY indices of antioxidant proteins under study indicated the possibility of better interaction with water molecules.

Functional Characterization

The SOSUI server classified DHAR and 2-CPs antioxidant proteins as membrane proteins and rest of the other foxtail millet antioxidant proteins as soluble proteins. One transmmembrane region each has been identified in both of the membrane proteins. The transmembrane regions were rich in hydrophobic amino acids and the length of transmembrane regions varied from 15 a.a. (in 2-CPs) to 26 a.a. (in DHAR). Transmembrane regions with their length and type identified are presented in Table 4.

For functional characterization of antioxidant proteins, prediction of disulfide bridges, motifs, patterns, and secondary structure were performed. Disulphide bridges play an important role in determining thermo stability of antioxidant proteins. Number and position of cysteine residues present in foxtail millet antioxidant proteins, predicted by CYS-REC tool, are presented in Table 5. Number of Cys residues ranged from 1 to 3 however, none of the proteins contain disulphide bridges.

Prosite analysis suggested the functionality of these proteins with profiles and patterns identified for characteristic function and are presented in Table 6. The only antioxidant protein exhibiting presence of a pattern was APX, in which two types of patterns *viz*, Peroxiadse_2 and Peroxidasde_1 were identified. The profile found in this protein was Peroxidase_4 which is a plant heme peroxidase family profile. Another type of profile observed was GST_NTER in DHAR protein. Rest of the antioxidant proteins were shown to have Thioredoxin_2 type of profile. These motifs, typically around 10 to 20 amino acids in length, arise because specific residues and regions thought or proved to be important to the biological function of a group of proteins are conserved in both structure and sequence during evolution [15].

Secondary Structure Prediction

Various structural motifs are often closely linked to protein function. For this reason, when working with protein 3D structures, it is important to be able to recognize the different types of secondary structure elements. Secondary structure features of foxtail millet antioxidant proteins as predicted using SOPMA revealed that random coils and alpha helix dominated among secondary structure elements followed by extended strand and beta turns for all sequences. The results were represented in Table 7. The percentage of alpha helix and random coil were almost equal in case of proteins PRDX(XP_004951605.1) and APX. Whereas, the proteins PRDX

Table 2: Amino acid composition of foxtail millet antioxidant proteins.

Amino acid	PRDX (XP_ 004969519.1)	PRDX (XP_ 004951605.1)	CYS PRX	APX	DHAR	2-CPs
Ala (A)	10.5%	15.0%	8.1%	10.8%	9.9%	13.5%
Arg (R)	1.9%	6.6%	5.4%	4.0%	4.0%	5.0%
Asn (N)	2.5%	1.3%	2.7%	1.6%	2.2%	2.3%
Asp (D)	5.6%	5.7%	9.0%	7.6%	5.8%	6.5%
Cys (C)	0.6%	0.9%	1.4%	0.8%	2.6%	1.2%
Gln (Q)	4.9%	0.9%	1.4%	3.2%	1.8%	3.1%
Glu (E)	6.2%	4.8%	4.5%	6.8%	5.5%	5.0%
Gly (G)	9.9%	7.9%	7.7%	9.2%	5.8%	6.2%
His (H)	1.9%	0.9%	2.7%	2.8%	1.8%	1.2%
Ile (I)	5.6%	1.3%	5.9%	2.8%	4.7%	5.8%
Leu (L)	11.7%	12.3%	8.1%	11.2%	11.7%	8.1%
Lys (K)	7.4%	4.8%	7.2%	6.4%	6.9%	5.8%
Met (M)	1.9%	2.6%	2.3%	1.6%	1.5%	1.2%
Phe (F)	4.9%	3.5%	3.6%	5.2%	5.5%	5.4%
Pro(P)	4.3%	4.0%	7.2%	7.2%	7.7%	5.4%
Ser (S)	5.6%	9.7%	5.0%	6.0%	8.0%	8.5%
Thr (T)	4.9%	8.8%	6.8%	4.4%	5.8%	4.6%
Trp (W)	1.2%	0.4%	0.9%	0.8%	1.1%	0.8%
Tyr (Y)	1.2%	0.9%	2.7%	2.8%	2.6%	2.7%
Val (V)	7.4%	7.5%	7.2%	4.8%	5.1%	8.1%
% of Hydrophilic residues	45.7%	48.5%	52.2%	48.8%	49.6%	47.5%
% of hydrophobic residues	54.3%	51.5%	47.8%	51.2%	50.4%	52.5%

Table 3: Parameters computed using Expasy's ProtParam tool.

Antioxidant Proteins	Accession No.	Length	M.wt.	pI	-R	+R	EC	II	AI	GRAVY
PRDX	XP_004969519.1	162	17639.0	5.34	19	15	13980	43.65	99.38	0.069
	XP_004951605.1	227	23692.1	8.58	24	26	8542.5	42.21	89.96	0.072
CYS PRX	XP_004958464.1	221	24293.7	6.30	30	28	20002.5	35.59	83.85	-0.305
APX	XP_004958804.1	250	27160.6	5.18	36	26	21492.5	33.77	79.32	-0.322
DHAR	XP_004965063.1	274	30000.6	6.53	31	30	27117.5	39.75	88.72	-0.046
2-CPs	XP_004952670.1	260	28050.0	5.97	30	28	21492.5	34.31	90.88	0.018

Table 4: Transmembrane regions identified by SOSUI signal server.

Antioxidant proteins	Accession no.	Transmembrane region	Length	Type
DHAR	XP_004965063.1	MAILLRGTSAAAAATAGPSSTLLATT	26	Signal peptide
2-CPs	XP_004952670.1	MACSFAAATAVSSAP	15	Signal peptide

Table 5: Position of cysteine residues predicted by CYS_REC.

Antioxidant proteins	Accession No.	No. and Position of Cysteine residues
PRDX	XP_004969519.1	1 (51)
CYS PRX	XP_004958464.1	3 (46,72,148)
PRDX	XP_004951605.1	2 (113,138)
APX	XP_004958804.1	2 (32,168)
DHAR	XP_004965063.1	3 (33,59,73)
2 CPs	XP_004952670.1	3 (3,113,235)

(XP_004969519.1) and 2CPs showed quite high percentage of alpha helix followed by random coil. In rest of the proteins higher percentage of random coil was noticed.

Model building, Evaluation and Visualization

The modeling of the three dimensional structure of the proteins was performed by two homology modeling programs Geno 3D (Figure 1) and Swiss Model (Figure 2).

A comparison of the results obtained from the Geno 3D and Swiss Model (Tables 8 and 9) showed that the models generated by Swiss Model were more acceptable in comparison to that generated by Geno3D. The stereo chemical quality of the predicted models and accuracy of the protein model was evaluated after the refinement process using Ramachandran Map calculations computed with the help of PROCHECK program. The Ramachandran plot generated by PROCHECK for models constructed by Geno 3D and Swiss Model are represented in Figure 3 and 4 respectively.

In the Ramachandran plot analysis, the residues were classified according to its regions in the quadrangle. The red regions in the graph indicate the most allowed regions whereas the yellow regions represent allowed regions. Glycine is represented by triangles and other residues are represented by squares. The result revealed that the modeled structure by Swiss Model for PRDX, CYS PRX, APX,

Table 6: Prediction of Patterns and Profiles by PROSITE.

Antioxidant Protein	Accession no.	Patterns found and their Position in Protein	Profiles found and their Position in Protein
PRDX	XP_004969519.1	-	THIOREDOXIN_2 (Thioredoxin domain profile), 4-162
	XP_004951605.1	-	THIOREDOXIN_2 (Thioredoxin domain profile),65-227
CYS PRX	XP_004958464.1	-	THIOREDOXIN_2 (Thioredoxin domain profile), 4-165
APX	XP_004958804.1	PEROXIDASE_2 (Peroxidase active site signature), 33-44 PEROXIDASE_1 (Peroxidase proximal heme-ligand signature), 155-165	PEROXIDASE_4 (Plant heme peroxidase family profile), 74-250
DHAR	XP_004965063.1	-	GST_NTER (Soluble glutathione S-transferase N-Terminal domain profile), 63-141
2-CPs	XP_004952670.1	-	THIOREDOXIN_2 (Thioredoxin domain profile), 67-226

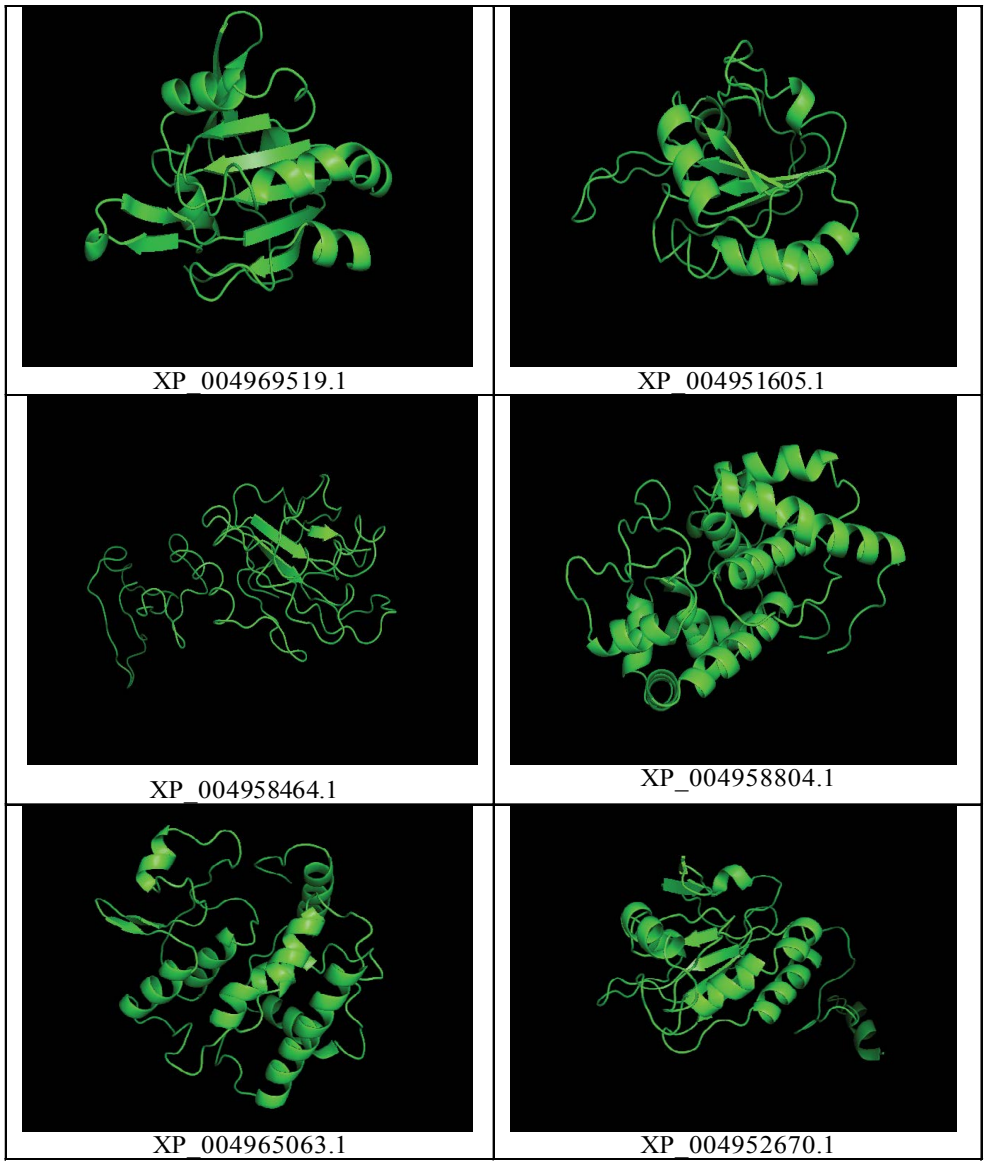

Figure 1: Models of Antioxidant proteins of Foxtail millet by GENO 3D software.

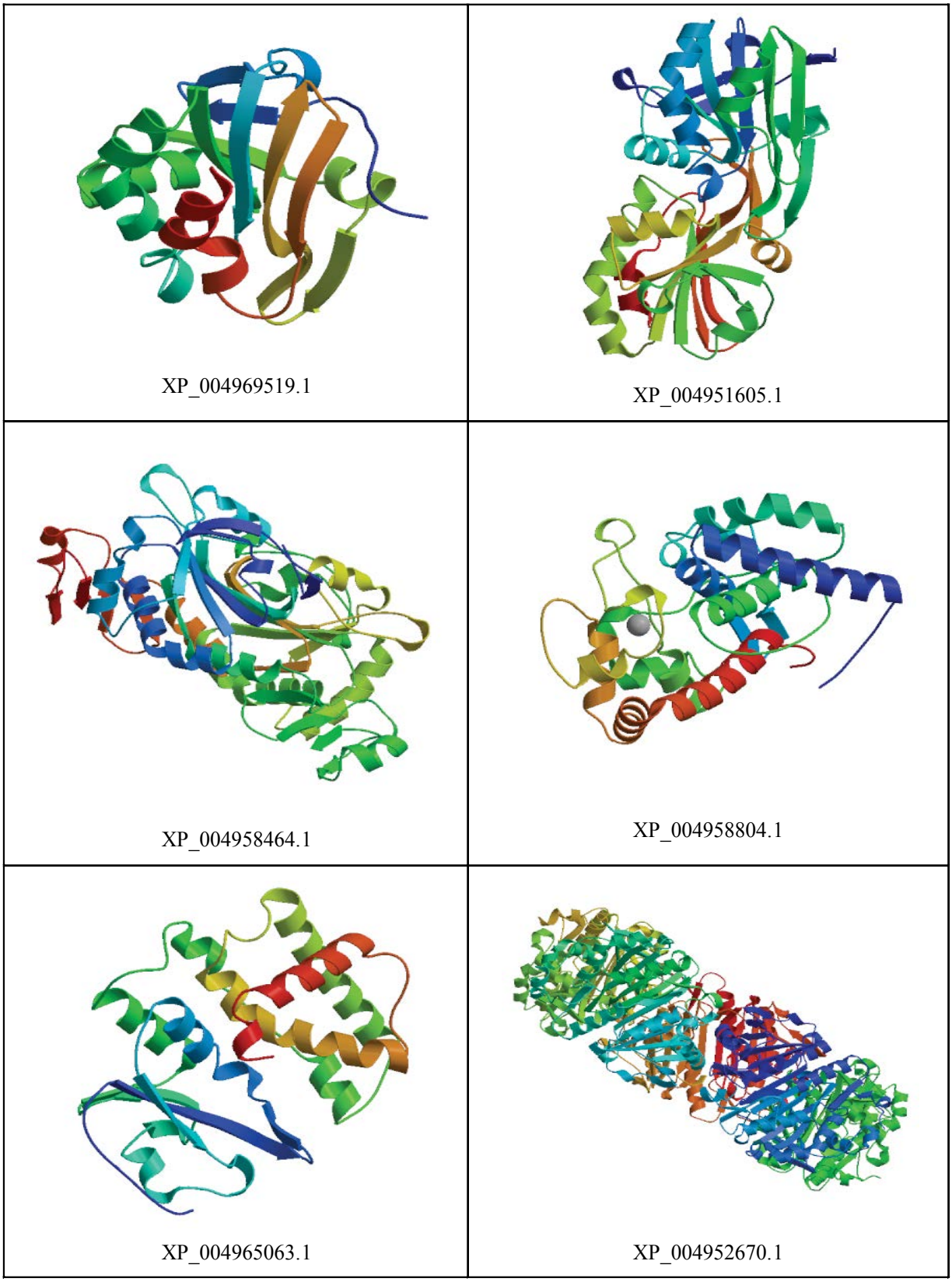

Figure 2: Models of Antioxidant proteins of Foxtail millet By Swiss Model.

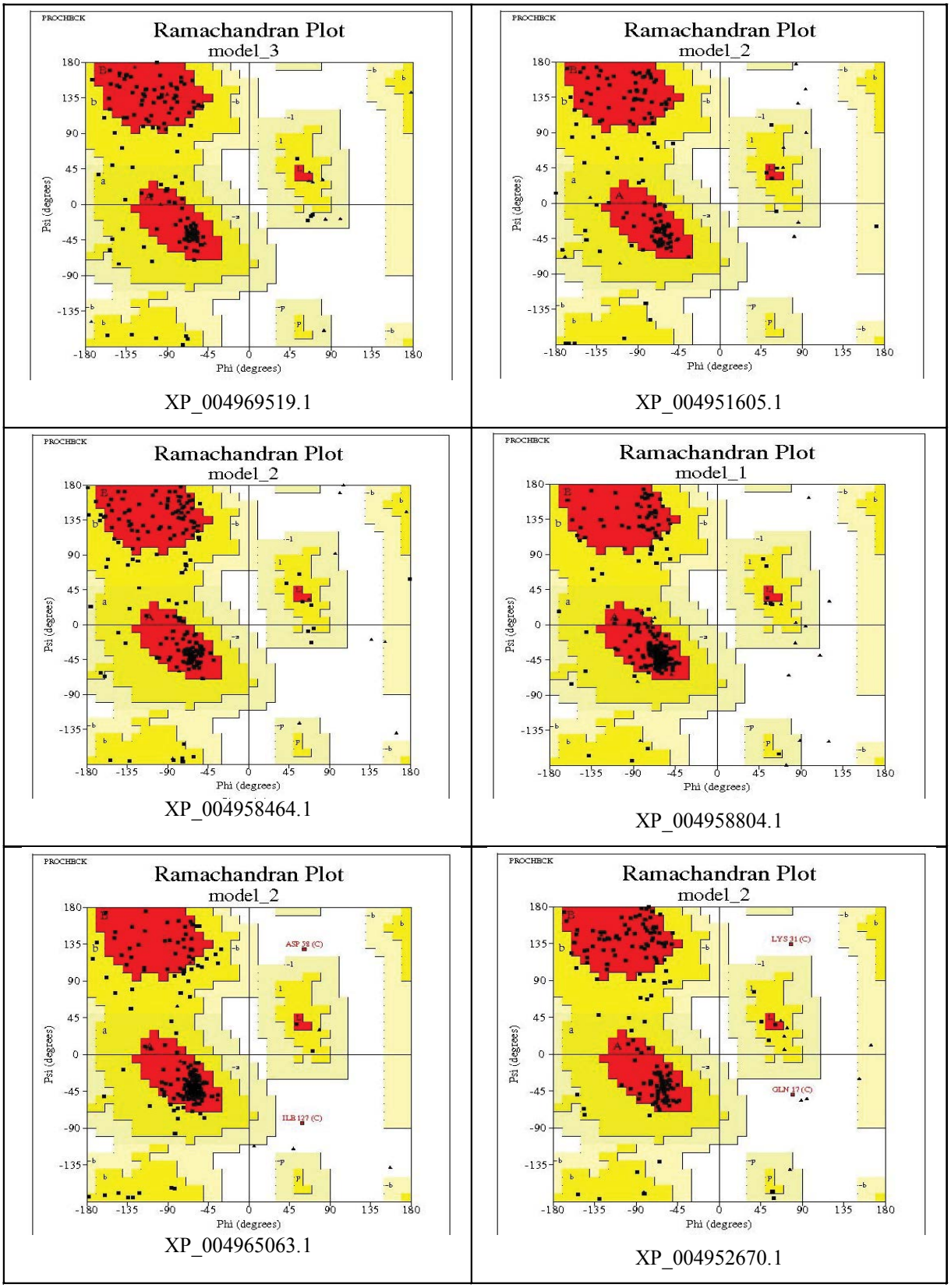

Figure 3: Ramchandran plot of the Geno 3D modeled antioxidant proteins by PROCHECK.

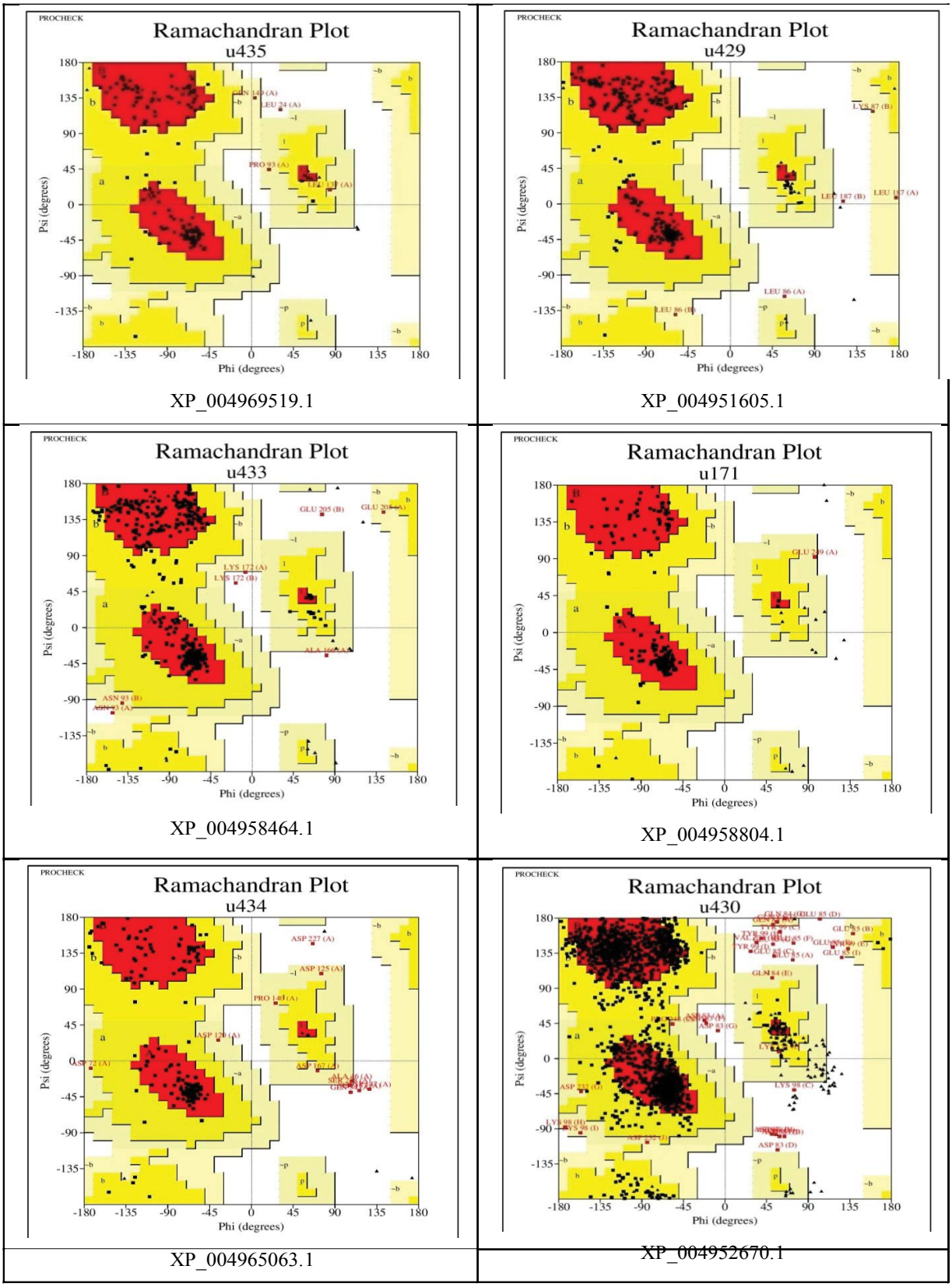

Figure 4: Ramchandran plot of the Swiss modeled antioxidant proteins by PROCHECK.

Table 7: Secondary structure prediction by SOPMA.

Antioxidant proteins	Alpha helix	Extended strand	Beta turn	Random coil
XP_004969519.1	34.57%	22.84%	15.43%	27.16%
XP_004951605.1	34.80%	21.59%	10.13%	33.48%
XP_004958464.1	26.24%	23.53%	11.76%	38.46%
XP_004958804.1	40.40%	12.40%	8.80%	38.40%
XP_004965063.1	28.83%	24.45%	9.12%	37.59%
XP_004952670.1	46.15%	16.92%	6.54%	30.38%

Table 8: Ramachandran plot calculation and comparative analysis of the models from Geno 3D and Swiss-model computed with PROCHECK program.

Servers	**Geno 3D (Residues in the regions)**				**Swiss Model (Residues in the regions)**			
Antioxidant	**a**	**b**	**c**	**d**	**a**	**b**	**c**	**D**
XP_004969519.1	76.6%	20.4%	2.9%	0.0%	88.2%	9.6%	1.5%	0.7%
XP_004951605.1	75.0%	19.1%	5.9%	0.0%	87.9%	10.3%	1.1%	0.7%
XP_004958464.1	77.3%	20.5%	2.2%	0.0%	84.5%	13.6%	0.5%	1.4%
XP_004958804.1	86.0%	13.5%	0.5%	0.0%	92.2%	7.3%	0.5%	0.0%
XP_004965063.1	77.7%	21.1%	0.0%	1.1%	84.1%	10.2%	2.8%	2.8%
XP_004952670.1	81.9%	15.7%	1.2%	1.2%	82.7%	15.4%	0.7%	1.2%

Table 9: RMS Z-score for bond angles of modeled protein structure using WHAT IF.

Antioxidant protein	**Accession no.**	**RMS Z Score for bond angles**
PRDX	XP_004969519.1	1.198
	XP_004951605.1	1.180
CYS PRX	XP_004958464.1	1.295
APX	XP_004958804.1	0.906
DHAR	XP_004965063.1	1.480
2-CPs	XP_004952670.1	0.933

DHAR, and 2CPS has 88.2%,84.5%,92.2%,84.1% and 82.7% residue respectively in most favored region. The distribution of the main chain bond lengths and bond angles were found to be within the limits for these proteins. Such figures assigned by Ramachandran plot represent a good quality of the predicted models by Swiss Model. Sarika et al. [16] and Thakur and Hande [17] reported the same results in legume and barley, respectively.

The modeled structures of Foxtail millet antioxidant proteins were also validated by other structure verification servers WHAT IF. Standard bond angles of the six models constructed by Swiss Model were determined using WHAT IF. The analysis revealed RMS Z-scores were almost equal to 1 and higher than 1 suggesting high model quality. Thus we can conclude that the homology modeling is considered as the most reliable computational technique for deciphering the 3D structures in the absence of crystal structure of these antioxidant proteins. The postulations made in this study may be confirmed experimentally using X-ray crystallography or NMR spectroscopy for better understanding the functional mechanism and molecular biology of these antioxidant proteins.

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