

Modeling and phylogenetic analysis of chloroplast ascorbate peroxidase in *Ipomea nil* (L.)

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Abstract: Ascorbate peroxidase (APX) is one of the major antioxidant enzymes involved in the detoxification of hydrogen peroxide. APX is found in chloroplast, cytosol and microsomes. The structural model for the APX isozymes has not been solved. In the present study, we describe the structural model for the chloroplast APX of *Ipomea nil* (morning glory) using homology model, which provides an insight for better understanding of APX molecular function in providing oxidative stress tolerance in plants.

Keywords: *Ipomea nil*, Ascorbate peroxidase, Structure, Phylogenetic analysis.

I. INTRODUCTION

Plants are frequently exposed to a wide variety of environmental stresses such as drought, salinity etc. which invariably reduces their growth and productivity^[11]. One of the most common outcome of the environmental stress is the synthesis of reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) or a hydroxyl radical (OH) either by the univalent reduction of O_2 , or by the dissipation of excess light energy^[15]. These ROS formed are highly deleterious to plants either by quenching the cell organelles or by reacting among themselves. Superoxide radicals formed can be more threatful as they cannot pass through the cell membrane thereby accumulates in cells forming excess of hydroxyl radicals by Fenton reaction which can eventually lead to death of cells^[8]. Plant system possesses a variety of enzymatic and non-enzymatic defense systems to minimize the deleterious affects of ROS. The non-enzymatic component includes antioxidants such as α -tocopherol, carotenoids, ascorbate and glutathione that are free-radical-scavenging molecules. The enzymatic component contains enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase respectively^[12]. Superoxide radicals are dismutated by the concomitant activities of super oxide dismutase (SOD), leading to the formation of hydrogen peroxide (H_2O_2), via the metal catalyzed Haber-Weiss-type reaction, which can pass through the cell membranes. Successful elimination of hydrogen peroxide is achieved by a cascade of enzymes expressed in different organelle of the cell such as catalase (CAT) in the peroxisomes and ascorbate peroxidase (APX) in the chloroplasts, utilizing ascorbic acid (AA) as substrate^[13].

Ascorbate peroxidases are the enzymes belong to class I of the superfamily of bacterial peroxidases, fungal peroxidases and plant peroxidases. Eight isoforms of APX have been identified in *Arabidopsis thaliana* based on their cell organelle specificity: three cytosolic (APX1, APX2, APX6), two chloroplast types (stromal sAPX, thylakoid tAPX), and three microsomal (APX3, APX4, APX5). All the isoforms have a common origin but possess different values of molecular weight, stability, optimal pH, substrate specificity, localization and level of response to specific stress conditions due to the process of natural selection^[6]. APX1 detoxifies peroxidases such as hydrogen peroxide using ascorbate as substrate. Expression of APX2 is limited to bundle sheath cells of leaves when exposed to excess sunlight^[3]. Peroxisomes possess the

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capacity to release the signaling molecules such as O_2^- , H_2O_2 and nitric oxide, which are involved in signal transduction pathways into the cytosol and play an important role in regulating responses to abiotic stresses. Both tAPX and sAPX are important to reduce O_2^- to water in a two-step reaction catalyzed by superoxide dismutase (SOD) and APX.

Though many experiments have been carried out on various APX genes, only the structure of APX1 is best known. APX1 is studied to contain 9 introns having high content of A and T nucleotides. The first intron is in an unusual position in the 5'UTR region in mRNA^[6]. In species in which sAPX and tAPX are formed by alternative splicing of one gene (e.g., in spinach), chloroplast APX contains 12 introns. Such genes have a conserved SRE sequence (splicing regulatory cis-element), which helps to control alternative splicing^[17]. The role of ascorbate peroxidase in deteriorating the toxicity of H_2O_2 has been studied in many plants but the critical role of these isoenzymes in antioxidant depletion has not been studied. The present investigation is carried out to determine the structure model of chloroplast APX in *Ipomea nil* (Morning glory) which is having the close relationship with the *Ipomea batatas* (sweet potato) a sensitive species and *Ipomea biloba*, a highly resistant species, psammophyte capable of growing on the shores of Bay of Bengal.

II. MATERIALS AND METHODS

A. Identification of APX genes

Gene categories of *Arabidopsis thaliana* which code for chloroplast ascorbate peroxidase, chloroplast AT4G08390.1 was identified from TAIR (The *Arabidopsis* Institute Of Research) for full length genomic and cDNA sequences. Corresponding genes for chloroplast APX were identified in *Ipomea* using DFCI (The computational Biology and Functional Genomics Laboratory at Dana-Farber Cancer Institute and Harvard School of Public Health). Homology search was carried out, through DFCI BLAST, for cDNA sequence of *Arabidopsis* with morning glory. To further narrow down the prediction studies for accuracy only the ESTs who's ORF shows best fit in the ORF of the corresponding APX are selected. EST ID of chloroplast APX in *Ipomea* is TC 6532. Frames of the ORF of selected EST were confirmed by the above said procedure.

B. Phylogenetic analysis of the sequences

Alignment of all sequences was performed using Clustal W^[4]. Phylogenetic trees were plotted using MEGA software with UPGMA method. *Ipomea* ESTS separating along with *Arabidopsis* genes were pooled and their amino acid pattern analyzed by using box shade server.

C. Secondary structure prediction of APX genes

Secondary structure of the APX isoforms was predicted using PSIPRED online server by submitting the sequences *Ipomea* in FASTA format as input sequences^[9].

D. 3D structure Prediction

To determine the exact binding site 3D LIGAND SITE was used to predict the 3D structure of the protein.

E. Model evaluation

In structural biology recognition of errors in experimental and theoretical models of protein structures is a major problem. Evaluation of a models predicted insilico is a highly essential task because there is a possibility for errors at higher degree due to trivial and non-trivial mistakes. Inorder to enhance the accuracy, evaluation of the above predicted APX models was performed using PROSA web server.

III. Results and Discussion

Proteomics is not only oriented with the global analysis of the protein complex mixtures but also on global changes in protein expression in tissues in a natural or induced disease state (expression proteomics), the characterization of protein

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complexes, structures and functions of proteins^[14]. In the present study the specific structure of the stromal chloroplastic APX gene has been predicted and its model has been studied to identify the similarities in its functions with respect to the Arabidopsis APX genes. Using the BLAST search, the protein sequences homologous to *Ipomea* chloroplast APX were identified in petunia, orange, lettuce, soybean, maize, rice, wheat and *Arabidopsis thaliana*. For evaluating the phylogenetic relationship, the selected sequences were aligned using alignment explorer in Mega 4.0 with default parameters. Unrooted phylogenetic tree of 8 APX sequences was constructed by the UPGMA method in Mega 4 program. The level of confidence was estimated using bootstrap of 1000 replications, which are indicated against each branch. Phylogenetic analysis showed one large cluster comprised of *Ipomea*, petunia, Soybean and orange. Lettuce, maize, wheat, rice and Arabidopsis formed separate clades with higher boot strap values ranging from 0.05764-0.07616 respectively (Figure 1). The tree showed a clear difference in the clustering of two flowering plants *Ipomea* and petunia with minimum boot strap values followed by the independent clusters of all cereal crop plants maize, rice, wheat (Figure 1).

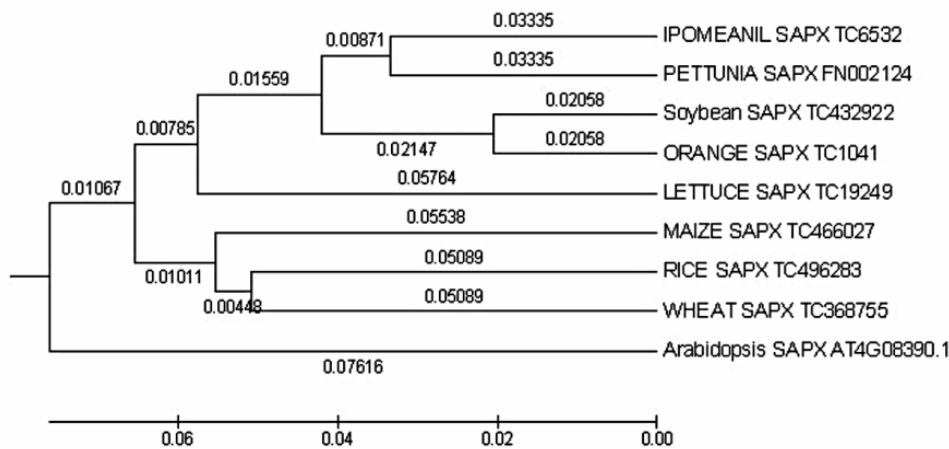
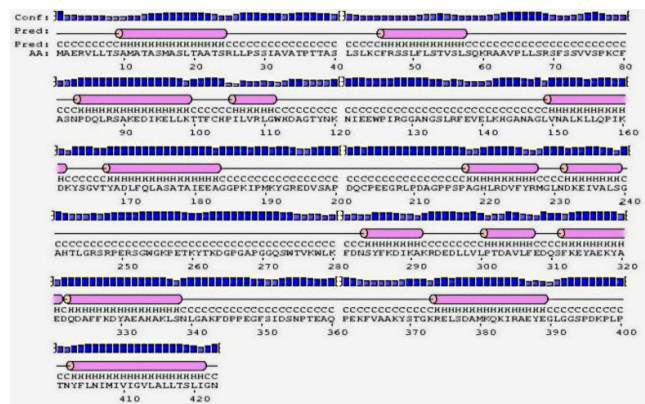


Fig. 1 Phylogenetic analysis of *Ipomea nil* chloroplast APX

The length of *Ipomea* chloroplast APX gene is 423 amino acids. The secondary structure of the protein was predicted which was divided into 13 alpha helices and 14 coils with two helices in both C and N terminal ends (Figure 2).



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Fig. 2 Secondary structure prediction of *Ipomea nil* chloroplast APX

No strands were identified in this structure. The confidence of prediction was high in the helix regions than that of coils. The components of secondary structure were depicted in Table 1.

Table 1
Structural components present in the APX protein of *Ipomea nil*

Structure	Amino acids
Coil	1-9, 25-45, 58-83, 100-105, 112-149, 162-167, 184-217, 228-231, 240-283, 292-300, 308-311, 322, 339-373, 391-402, 422-423
Helix	10-24, 46-57, 84-99, 106-111, 150-161, 168-183, 218-227, 232-239, 284-291, 301-307, 312-321, 323-338, 374-389, 403-421
Strand	Nil

The predicted model was further explored for identification of ligand binding sites which may be useful to understand specific role of functional site residues during catalysis (Table 2).

Table 2
Predicted binding sites of *Ipomea nil* Chloroplast APX

Residue	Amino acid	contact	Avg distance	JS divergence
105	PRO	25	0.14	0.69
108	VAL	24	0.68	0.51
109	ARG	25	0.35	0.81
112	TRP	25	0.00	0.84
210	PRO	25	0.00	0.83
211	ASP	25	0.01	0.37
212	ALA	25	0.02	0.59
221	LEU	25	0.00	0.60
225	PHE	25	0.36	0.83
238	LUE	25	0.02	0.75
241	ALA	25	0.01	0.62
242	HIS	25	0.00	0.91
244	LEU	25	0.14	0.52
245	GLY	25	0.00	0.74
246	ARG	25	0.00	0.74
247	SER	25	0.00	0.50
248	ARG	25	0.00	0.64
251	ARG	25	0.01	0.46
252	SER	25	0.00	0.44
254	TRP	25	0.40	0.66
274	TRP	25	0.06	0.65
300	LEU	15	0.72	0.68
302	THR	25	0.40	0.67
330	TYR	24	0.50	0.77

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Proteins are the most complex chemical entities in nature comprising of a large number of atoms, variable composition, convoluted topology and complex surface features which make simple descriptions of protein structures almost impossible [7]. The ‘indescribable nature’ of proteins also makes it very difficult to quantitatively assess the quality or correctness of an experimentally determined protein structure. With increase in the number of genomes sequenced and more amount of genes are being deposited in the databases there is an increase in the need for the molecular analysis to identify the function of the proteins which can be applicable to the proteins groups which are formed based on their interactions. In the present study three-dimensional models of the *Ipomea* chloroplast APX protein were predicted using 3D-ligand site, which displayed an excellent global and local stereochemical properties (Figure 3a). Single linkage clustering has been used to group the ligands and the selection of the ligand was based on the prediction of binding site formation. Residue conservation was calculated using the Jensen Shannon divergence score [2]. Using this application the entire structure was coloured from sky blue to dark blue indicating the increasing intensity of divergence score. Labeled 3d structure also contained heterogens, which were described to be non-standard residues, prosthetic groups, inhibitors, solvents (except water), or ions that are unknown to the PDB. In this structure the heterogen was indicated in the center (Figure 3b).

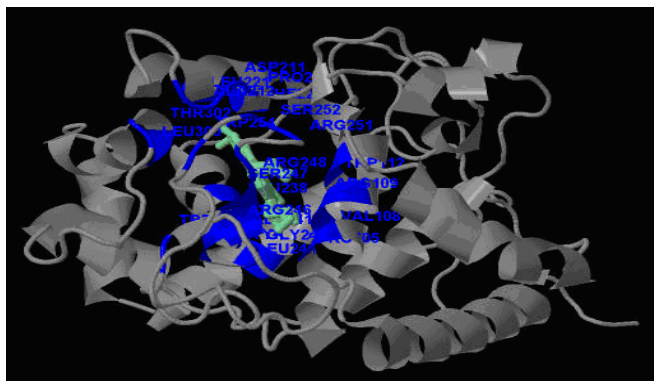


Fig.3a 3D Structure of *Ipomea nil* chloroplast APX predicted ligand binding sites

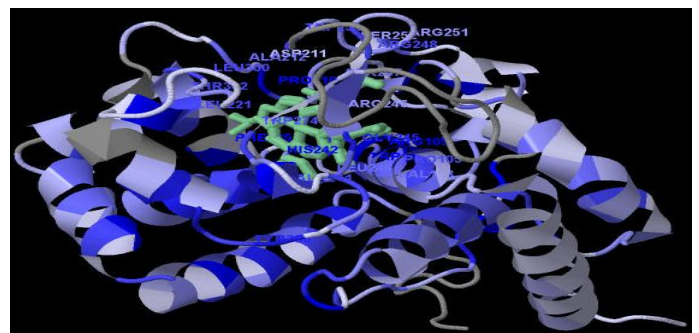


Fig.3b Jensen & Shannon divergence indication in *Ipomea nil* chloroplast APX 3D Structure

Z-score value, a measure of model quality as it measures the total energy of the structure was predicted using PROSA server. The Z score value of the structure was -8.94 (Figure 4) and all residues were lying within the allowed regions of the Ramachandran plot (Figure 5). In the plot, β core is in between -170 and -40 of ϕ region comprising of more than 50 residues where as in the favorable region only 10 residues were observed. α R region consisting of most residues whose core is diagonal. Due to local backbone interactions, α R region is diagonal in shape. This can also be due to the disfavoring of conformations near -130° and -30° of the plot [1]. Glycine residues were present in both generous and allowed regions with only one normal residue in allowed region. The structure of *Ipomea* chloroplast APX is having continuous right helices than left and more over α R is over populated indicating that structure might have less conformation

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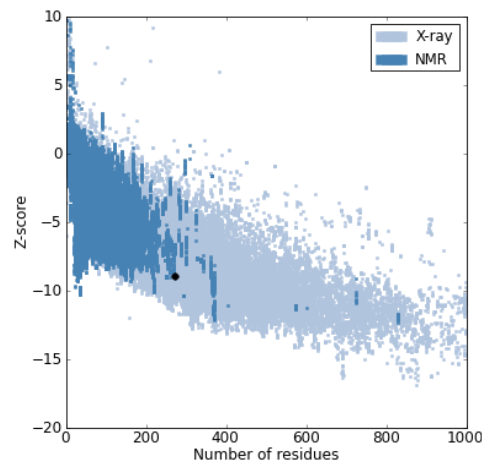


Fig. 4 PROSA web analysis for modeled *Ipomea nil* chloroplast APX

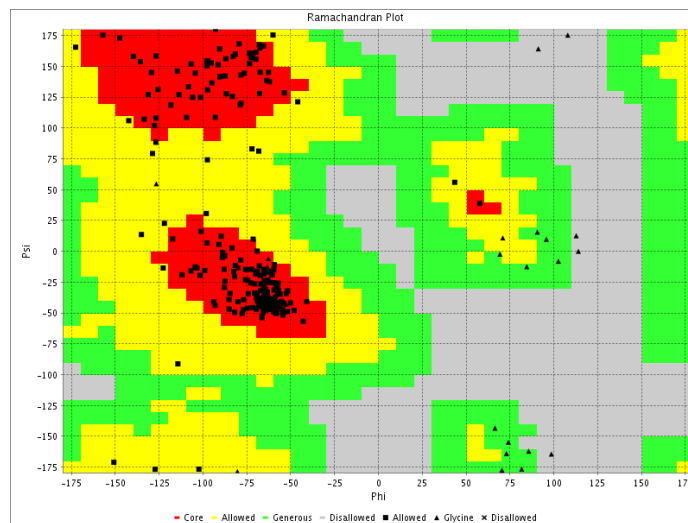


Fig.5 Ramachandran plot for the *Ipomea nil* Chloroplast APX structure

Continuous repetitions in the region of -57° ϕ values suggest that this is a regular right handed helical structure, which is rigid in nature as revealed by the clustered residues in the β and α right handed regions (Figure 5). Number of residues in the core region are 266 and number of residues in the allowed region are 2. In the generous region, 9 residues are found to be present, among which most of them are glycine. 5 glycine residues are in the outlier region and approximately 54 residues are in β core region. The α helix right region accommodates maximum residues in the structure indicating helical domination in this structure. Repetitive residues with ϕ and ψ values between the 175 and -55 indicates that the structure is

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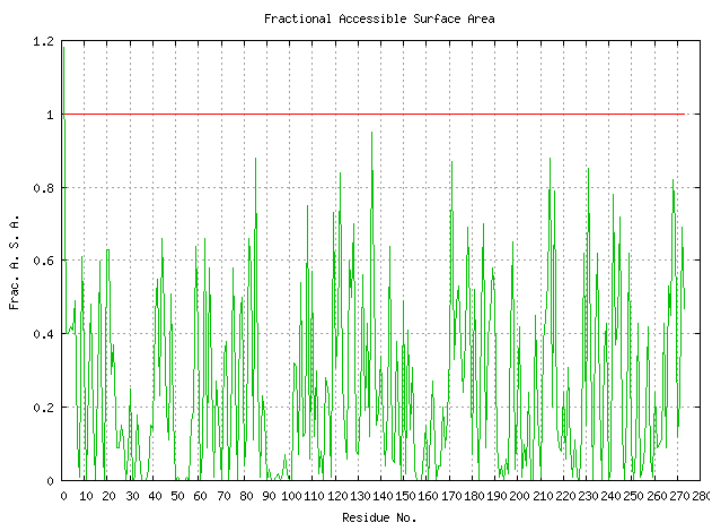
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a regular one. The above analysis about the predicted structure provides an evidence that the predicted 3D structure of *Ipomea* chloroplast APX is of good quality.

The accessible surface areas in the amino acid sequence of *Ipomea* chloroplast APX were predicted using the VADAR servers for the model prediction (Figure 6). We have predicted the ASA of each amino acid in the sequence along with the fractional residual volume available for the amino acids in the main as well as in the side chains. The quality of the model with respect to the stereo packing and 3D profile quality also predicted using the VADAR server.

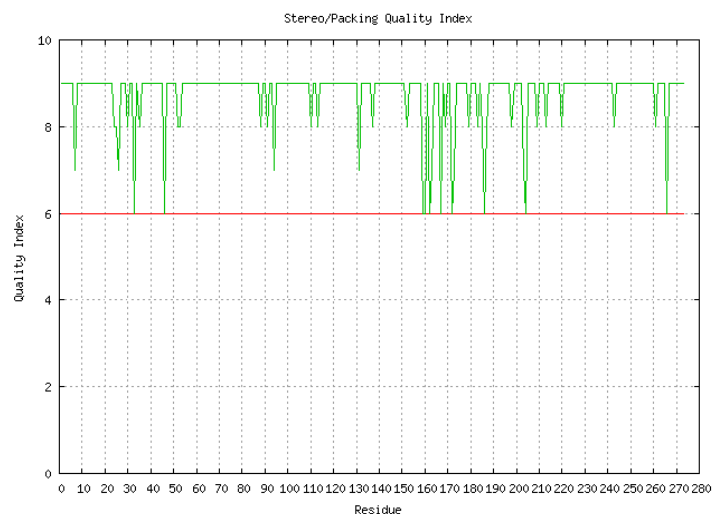
The area accessible to the water molecule on the protein structure is said to be Accessible Surface Area (ASA) which was measured in the square angstroms or as fractional ASA ranging from 0 to 1. Hydrophilic residues occupied a large fraction of ASA with hydrophobic residues in a small fraction (Figure 6a). ASA values both for the whole structure and side chains were predicted using VADAR, with more than 40 residues of the *Ipomea* chloroplast APX have ASA scores less than 0.8, indicating a good folding and the structure is not accessible to water molecules. The excluded volume of the structure was measured using VADAR represented as the Residue Volume (RV), measured in cubic angstroms. The VADAR of *Ipomea nil* chloroplast APX indicates that the residues 25,30,32,33,61,65,89,91,93,130,160 to 172, 203,209,215 and 261 have RV scores 1.25 (Figure 6b), indicating that most of the residues are located in the interiors. The stereo chemical/packing quality index of the VADAR prediction categorizes phi/psi and omega trends according to the criteria given by Morris et al. (1992)^[10]. It also includes the presence of packing defects mostly large cavities or atomic overlaps as part of the quality score. These indices also allow specific problem residues to be rapidly identified. In the present study the quality index of *Ipomea* chloroplast APX structure was estimated to be 6 indicating no major large cavities or overlaps in the predicted structure (Figure 6c). Quality index model of *Ipomea* chloroplast APX gives quality index of each and every residue in the structure for side chain misfolding, stereo chemical overlaps, insufficient atom packing etc.,^[16]. In this structure, the overall model quality is good which can be read by the high peaks reaching a score value of 8. However, the residues lying in between 40 to 60 are below the mark with score values of less than 4 (Figure 6D).



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6a) Fractional Accessible Surface Area

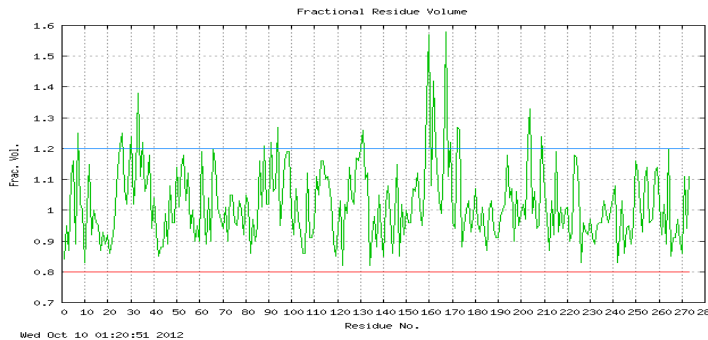
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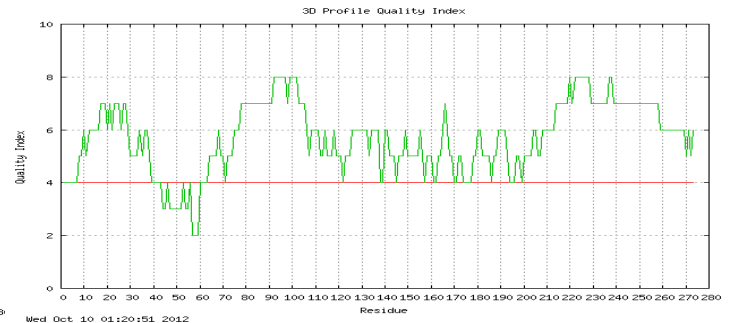
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6c) Stereo Packing Quality Index

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6b) Fractional Residue Volume



6d) 3D Profile Quality Index

IV. CONCLUSIONS

The structure of chloroplast APX gene of *Ipomea* is important for understanding its molecular function in response to oxidative stress. The structural model of *Ipomea* chloroplast APX using homology modeling has been described in the present study, which provides an insight of molecular function towards the understanding of oxidative stress tolerance in plants. Further studies are in progress for comparative analysis of all APX isozymes in *Ipomea* in comparison with *Arabidopsis* genes.

ACKNOWLEDGMENTS

The research lab of Dr.KVC is supported by funding from the grants of DST, DBT and UGC, Govt. of India. GVR acknowledges DST for the research fellowship.

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