Online: ISSN 2771-5868
Print: ISSN 2771-585X

## Original Research Article

# *In Silico* Structural Analysis of Chlorophyll Synthase: A Comparative Study between Monocot and Eudicot

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Received: September 09, 2021; revised: January 15, 2022; accepted: January 20, 2022

https://doi.org/10.17605/OSF.IO/6Q2JU

**Abstract:** Chlorophyll, a member of an essential class of green pigments, is predominantly found in all mature plant cells, whether in the leaf of a green plant or any photosynthetic organisms, including cyanobacteria or algal species. The primary function of this pigment is to convert the light energy into chemical energy through a series of synthesizing several chemical compounds; alternatively, the whole process is termed as 'Photosynthesis.' Monocots and eudicots are two different variants of angiosperms or flowering plants, where this chlorophyll synthesis occurs distinctly. Our present course of study mainly aims at the *in silico* comparative structural analysis of chlorophyll synthase enzyme in these two divergent angiosperm species.After retrieving the FASTA sequence for each plant chlorophyll synthase from the UniProt database, Multiple Sequence Alignment followed by Phylogenetic Tree preparation was done using CLUSTALW; the primary and secondary structural components analyses were done by ExPASy ProtParam and GOR4 server; after that 3D structure of each of the protein sequence was generated via Homology Modelling using SWISS-MODELonline server. Lastly, each of the protein models was undergone through various quality checking parameters. Multiple Sequence Alignment followed by Phylogenetic Tree prediction and 3D structure superposition analysis reveals that though the chlorophyll synthases of these two divergent angiosperm species evolved in different time scales, they share highly conserved structure. Since the structure is the basis of function, the findings support the overall functional importance of the terminal enzyme of the chlorophyll synthesis pathway.

**Key words:** Chlorophyll Synthase; Eudicot; Homology Modelling; Monocot; Phylogenetic Analysis

## Introduction

Chlorophyll synthase belongs to 'transferases,' which transfers the polyprenyl group. E.C. number 2.5.1.62 (Schomburg, D., Schomburg, I. and Chang, A., 2013). Therefore, chlorophyll synthase is also known as chlorophyll synthetase. The enzyme also has an alternative name, i.e., polyprenyl transferase. There is one inhibitor named N-phenylmaleimide that inhibits the activity of this enzyme. On the other hand, metal ions like $Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$ are required to reconstitute activity after treatment with EDTA (another type of inhibitor).

Chlorophyll arose from an evolutionarily conserved biosynthetic pathway of tetrapyrrole, consisting of at least 18 different enzymatic steps. However, the basic steps from the early precursor to chlorophyllide were evolved only once. The chlorophyll-synthesizing pathway can be described in three different subdivisions: (1) the formation of 5-aminolaevulinic acid, (2) the conversion of eight molecules of 5-aminolaevulinic acid into the tetrapyrrole molecule protoporphyrin IX and (3) the chelation of Mg2+ and the completion of Mg-porphyrin

to the lipophilic chlorophyll molecule(Cullen, E., Katherine, 2007). The main reaction procedure is given in Figure 1.

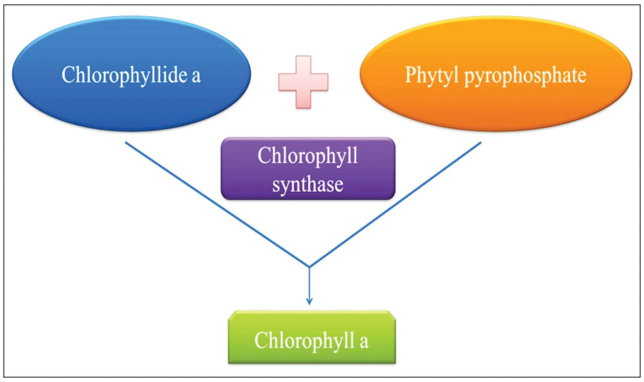


**Fig. 1.** The Reaction Procedure Mediated by Chlorophyll Synthase Enzyme.

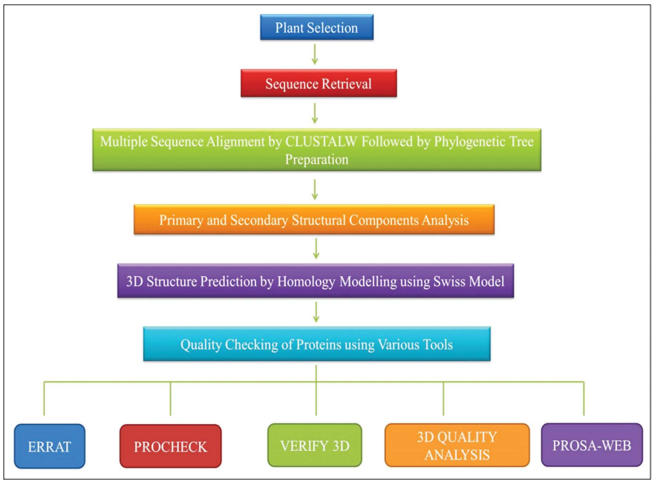


**Fig. 2.** The Overall Work Flow.

Chlorophyll synthase cannot synthesize chlorophyll a. The enzyme, instead, catalyzes the last step of chlorophyll biosynthesis, namely prenylation (esterification) of chlorophyllide with phytyl diphosphate or geranylgeranyl diphosphate (Bollivar, David W., 2007).

One important enzyme in chlorophyll biosynthesis or tetrapyrrole biosynthetic pathway, Protoporphyrinogen IX oxidase, is common for plants and humans. Protoporphyrinogen IX oxidase produces Protoporphyrin IX from Protoporphyrinogen IX, resulting in the formation of a 'haem' compound (one component of Haemoglobin).

Plants are the main source of food as well as oxygen. The green color of plants is due to the presence of a small molecule, 'chlorophyll,' inside it. Several types of chlorophyll pigments have been found, e.g., chlorophyll a, chlorophyll b, chlorophyll c, chlorophyll d, and chlorophyll f (Tanaka R and Tanaka A, 2011).The main enzyme involved in the chlorophyll synthesis pathway is chlorophyll synthase (Von Wettstein, D., 1995). Hence, this study aims to the comparative structural evaluation of chlorophyll synthase in different plants with the help of different bioinformatics and computational biology techniques. During work, we have selected seven plants belonging to both monocot and dicot origins for the *insilico* structural and evolutionary analysis of their chlorophyll synthesis key enzyme chlorophyll synthase. The overall workflow is represented in Figure 2.

## Materials and methods

### Plant selection

Plant taxonomy includes Kingdom, Phylum, Class, Clade, Order, Family, Genus, and Species.

According to Species 2000 & ITIS Catalogue of Life: 2017 Annual Checklist, the chosen plants (Table 1) are from the following taxonomic backgroundbelonging to the same phylum 'Angiosperm' (Chandra S, Chatterjee S, Bandyopadhyay S and Acharya K. 2014).

**Table 1.** Table Showing the Selected Plants Along With Their Scientific Names, Phylum and Class.

| Sl. No. | Plant Scientific Name | Phylum | Clade/Class |
|---|---|---|---|
| 1 | *Arabidopsis thaliana* | Angiosperm (Magnoliophyta) | Eudicotyledons (Magnoliopsida) |
| 2 | *Zea mays* | Angiosperm | Monocotyledons (Liliopsida) |
| 3 | *Oryza sativa* | Angiosperm | Monocotyledons |
| 4 | *Camellia sinensis* | Angiosperm | Eudicotyledons |
| 5 | *Nelumbo nucifera* | Angiosperm | Eudicotyledons |
| 6 | *Musa balbisiana* | Angiosperm | Monocotyledons |
| 7 | *Ananas comosus* | Angiosperm | Monocotyledons |

### Sequence Retrieval

Seven plant sequences were chosen for this research study. All seven primary sequences of chlorophyll synthase were retrieved from the UniProt KB Database website (https://www.uniprot.org/). Each plant name initials and their respective protein UniProt ID has been given in Table 2.

**Table 2.** Plant Name Initials Along With Respective Protein UniProt ID.

| Plant Scientific Name (initials) | Uniprot ID |
|---|---|
| AT | Q38833 |
| ZM | A0A1D6M2M3 |
| OS | Q5W6H5 |
| CS | A0A0B4SXX9 |
| NN | A0A1U7ZPJ8 |
| MB | E1UHJ5 |

### Primary and Secondary Structure Analysis

Primary and Secondary structures were analyzed through ExPASy ProtParam (https://web.expasy.org/protparam/) and GOR IV (https://npsa-prabi.ibcp.fr/NPSA/npsa_gor4.html) (Gasteiger E., Hoogland C., Gattiker A., Duvaud S., Wilkins M.R., Appel R.D. and Bairoch A., 2005) (Garnier, J, Gibrat, J. F, Robson B., 1996).

### Tertiary Structure Prediction

3D structures were predicted by Homology Modelling technique with the help of SWISS-MODEL server (https://swissmodel.expasy.org/) (Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., de Beer, T.A.P., Rempfer, C., Bordoli, L., Lepore, R. and Schwede, T., 2018).

Steps for Homology modeling are as follows –

i. Template Selection

ii. 3D Structure Prediction

### Tertiary Structure Quality Analysis

Structures were analyzed in the website named SAVES (https://saves.mbi.ucla.edu/) which consists of a few software, namely PROCHECK (Laskowski R A, MacArthur M W, Moss D S., and Thornton J M., 1993), Verify 3D (Eisenberg D, Luethy R., and Bowie J.U., 1997), ERRAT (Colovos C. and Yeates T.O., 1993), VADAR (http://vadar.wishartlab.com/) (Willard L, Ranjan A, Zhang H, Monzavi H, Boyko R, Sykes B., and Wishart D. S., 2003), as well as another website, i.e., ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php/) (Wiederstein M. and Sippl M.J., 2007). Then hydrogen bonds (H-Bonds) were added to those modeled structures.

PROCHECK analyses residue-by-residue geometry and overall structure geometry to determine the stereochemical quality of a protein structure. ProSA-web shows the output of the z-score. The *Z*-score indicates overall model quality.

### Total Energy Determination by Using Swiss-PDBViewer Software

Each newly generated 3D protein structure was undergone through Swiss-PDB Viewer (SPDBV) Software for calculating total energy in the form of KJ/ mol. The total energy was determined from the combined energy of bonds, angles, torsion, improper, non-bonded, and electrostatic constraints. The partial implementation of the GROMOS96 force-Field is used to conduct energy minimization. In this implementation, all computations are performed *in vacu*um, without the use of a reaction field.(Johansson, M.U., Zoete V., Michielin O., and Guex N., 2012).

### Multiple Sequence Alignment with Phylogenetic Tree Preparation

The sevenchlorophyll synthase amino acid sequences from different plant species were subjected to Multiple Sequence Alignment and thereafter followed by Phylogenetic Tree Preparation using CLUSTALW (https://www.ebi.ac.uk/) (Thompson J.D, Higgins D.G, and Gibson T.J., 1994).

## Results

### Primary structure analysis

The primary structural components and other protein parameters of each amino acid sequence have been analyzed using ExPASy ProtParam, and the result has been represented in Table 3.

### Secondary structural comparative analysis

Comparative secondary structural components have been done using the GOR IV online tool. From the results originated through the server, an evaluating graph(Figure 3)has been prepared to view the percentages of each component present in every protein.

### Tertiary structure prediction using homology modeling

Due to the unavailability of 3D structures of chlorophyll synthase enzyme in the PDB server, homology modeling has been performed using the SWISS-MODEL server. The 3D

**Table 3.** Table Showing Primary Structural Elements of Different Proteins

| Plant Name | No. Of Amino Acids | Molecular Weight | Theoretical pI | Total No. Of Positively Charged Residues | Total No. Of Negatively Charged Residues | Extinction Coefficients | Instability Index | Aliphatic Index | Grand Average Of Hydropathicity (GRAVY) |
|---|---|---|---|---|---|---|---|---|---|
| AT | 387 | 41881.44 | 8.52 | 30 | 27 | 83100/ 82850 | 27.04 | 104.81 | 0.267 |
| ZM | 362 | 38886.26 | 8.52 | 25 | 22 | 77140/ 76890 | 31.77 | 108.95 | 0.358 |
| OS | 376 | 40578.99 | 8.23 | 27 | 25 | 81610/ 81360 | 29.20 | 104.63 | 0.289 |
| CS | 374 | 40457.11 | 8.74 | 30 | 26 | 80120/ 79870 | 25.76 | 111.42 | 0.320 |
| NN | 395 | 42737.85 | 7.62 | 26 | 25 | 91580/ 91330 | 23.05 | 116.30 | 0.419 |
| MB | 398 | 44635.74 | 9.89 | 39 | 19 | 69370/ 68870 | 51.80 | 108.49 | 0.554 |
| AC | 383 | 41024.80 | 8.91 | 30 | 25 | 80120/ 79870 | 35.97 | 112.45 | 0.346 |

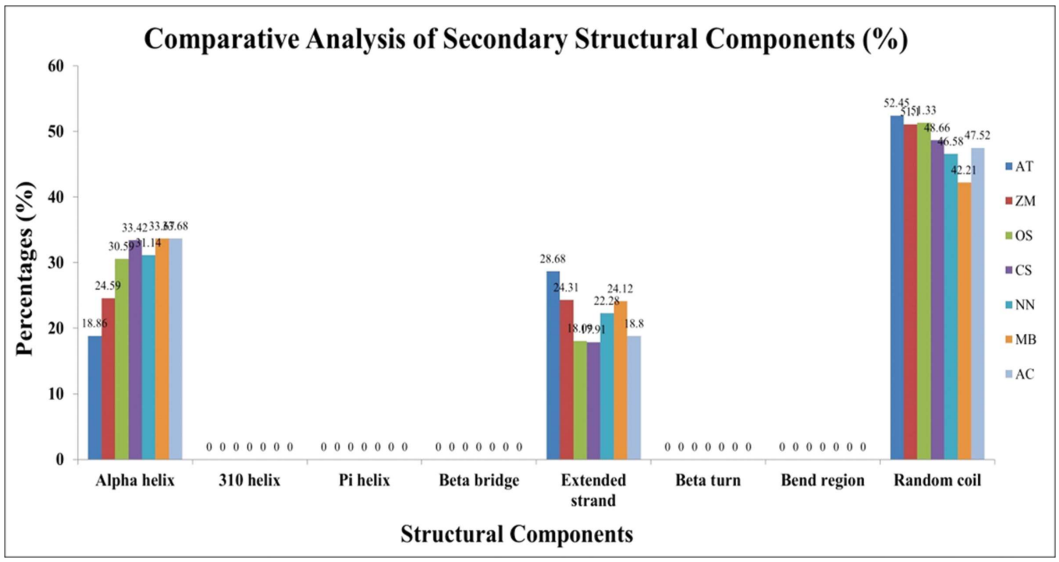


**Fig. 3.** A Comparative Graph Showing the Data of Different Secondary Structural Components.

structures generated through a series of threading, template selection, and many more parameters are given in Figure 4.

## Determination of Total Energy

Total energy determination of each protein was done via SPDBV and represented in a table form (Table 4).

**Table 4.** Table Showing Calculated Total Energy (KJ/mol).

| Plant Name | Total Energy (KJ/ mol.) |
|---|---|
| *Arabidopsis thaliana* | -5443.549 |
| *Zea mays* | -5166.650 |
| *Oryza sativa* | -5333.068 |
| *Camellia sinensis* | -5082.891 |
| *Nelumbo nucifera* | -5451.832 |
| *Musa balbisiana* | -8887.541 |
| *Ananas comosus* | -6274.881 |

## Tertiary structural quality analysis

The online server-generated 3D structures are undergone several quality analysis tests by various online tools to get an overall idea about those protein structures. Results from various tools are given in the following:

## Errat

The ERRAT score, i.e., the overall quality factor of the respective protein, is given in Figure 5. Here, the overall quality factor in the form of percentages generated in a graphical manner determines the nature of the proteins.

## Procheck

Ramachandran Plot generated from the SAVES6.0 server is given in Figure 6 and Table 5.

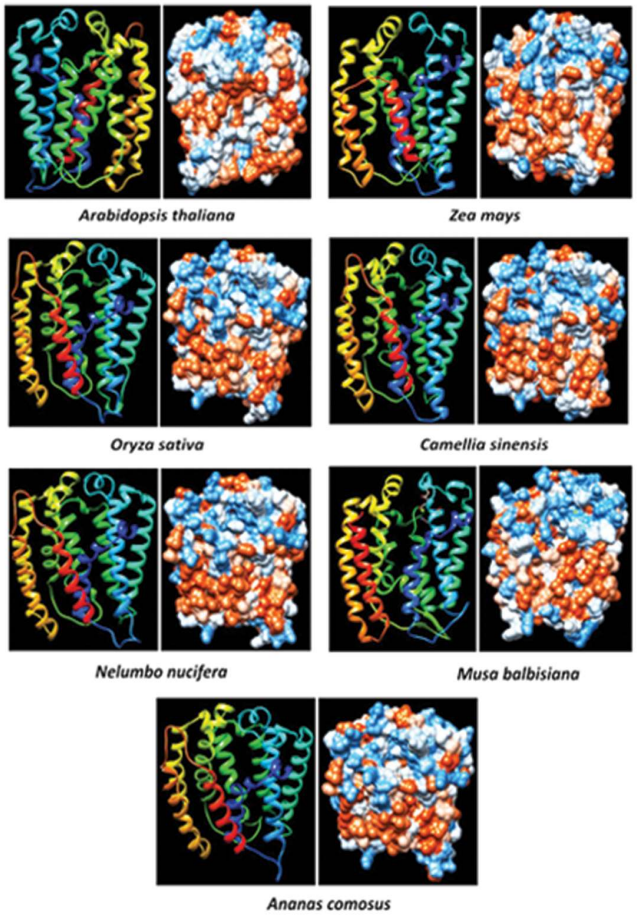


**Fig. 4.** The 3D Structures of Seven Chlorophyll Synthase Enzyme Belonging To Different Plants.

**Table 5.** Table Showing Ramachandran Statistics

| Plant Name | Residues (%) in additional allowed regions | Residues (%) in generously allowed region | Residues (%) in disallowed regions |
|---|---|---|---|
| *Arabidopsis thaliana* | 11.7 | 0.8 | 0.0 |
| *Zea mays* | 10.6 | 0.9 | 0.0 |
| *Oryza sativa* | 10.4 | 0.0 | 0.0 |
| *Camellia sinensis* | 11.8 | 0.4 | 0.4 |
| *Nelumbo nucifera* | 11.9 | 0.0 | 0.0 |
| *Musa balbisiana* | 8.6 | 0.4 | 1.2 |
| *Ananas comosus* | 12 | 0.0 | 0.4 |

## Verify-3D

Verify-3D result generated from the SAVES 6.0 server has been represented both in pictorial form (Figure 7) and tabulated form (Table6).

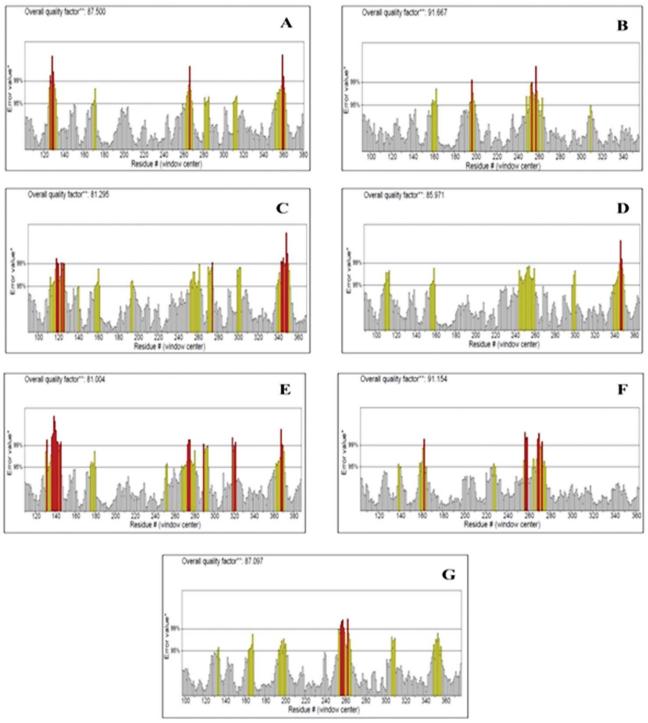


**Fig. 5.** The ERRAT Score of Seven Various Chlorophyll Synthase (A. *Arabidopsis thaliana,* B.*Zea mays*, C. *Oryza sativa*, D. *Camellia sinensis*, E.*Nelumbo nucifera*, F.*Musa balbisiana*, G.*Ananas comosus*)

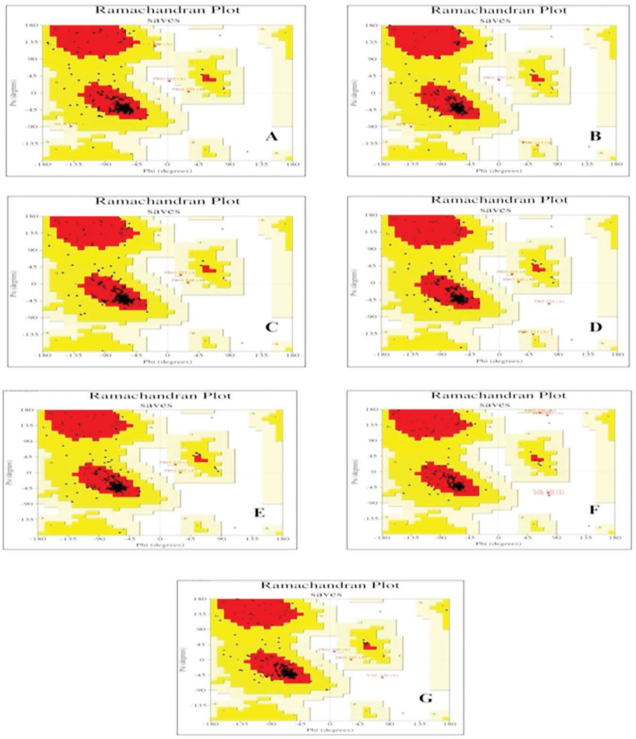


**Fig. 6.** The Ramachandran Plot of Seven Various Chlorophyll Synthase (A. *Arabidopsis thaliana,* B.*Zea mays*, C. *Oryza sativa*, D. *Camellia sinensis*, E.*Nelumbo nucifera*, F.*Musa balbisiana*, G.*Ananas comosus*)

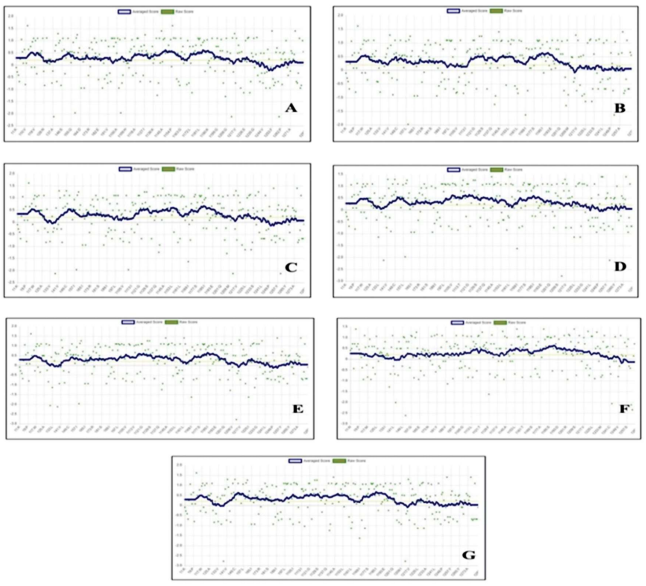


**Fig. 7.** The Verify 3D Results of Seven Various Chlorophyll Synthase (A. *Arabidopsis thaliana,* B.*Zea mays*, C. *Oryza sativa*, D. *Camellia sinensis*, E.*Nelumbo nucifera*, F.*Musa balbisiana*, G.*Ananas comosus*)

**Table 6.** Table Showing % of residues having 3d-1d score >= 2

| Plant Name | % of residues having average 3d-1d score >= 2 |
|---|---|
| *Arabidopsis thaliana* | 69.10 |
| *Zea mays* | 70.96 |
| *Oryza sativa* | 66.20 |
| *Camellia sinensis* | 74.48 |
| *Nelumbo nucifera* | 65.51 |
| *Musa balbisiana* | 66.42 |
| *Ananas comosus* | 66.20 |

## 3D Quality Analysis

3D Quality analysis results generated from the VADAR server have been represented in Figure 8.

## PROSA-WEB RESULT

Prosa-Web result for calculating Z scores is given in a tabulated manner (Table 7).

**Table 7.** Table Showing Prosa-Web Server Generated Z Score.

| | |
|---|---|
| Z-score (AT model) | -3.84 |
| Z-score (ZM model) | -3.17 |
| Z-score (OS model) | -3.34 |
| Z-score (CS model) | -3.88 |
| Z-score (NN model) | -3.84 |
| Z-score (MB model) | -4.19 |
| Z-score (AC model) | -4.38 |

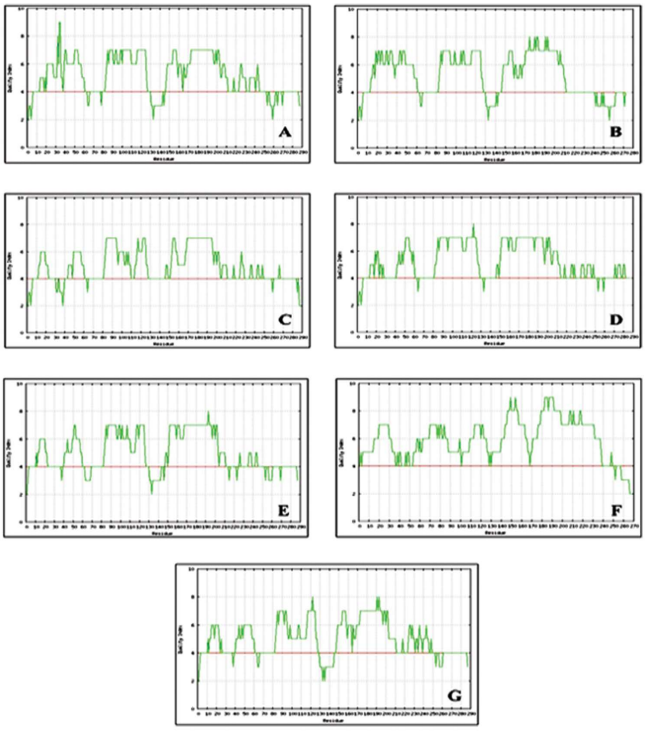


**Fig. 8.** The 3D Quality Analysis Results of Seven Various Chlorophyll Synthase (A. *Arabidopsis thaliana*, B.*Zea mays*, C. *Oryza sativa*, D. *Camellia sinensis*, E.*Nelumbo nucifera*, F.*Musa balbisiana*, G.*Ananas comosus*)


**Fig. 9.** Showing Results of Multilple Sequence Alignment Analysis by CLUSTALW

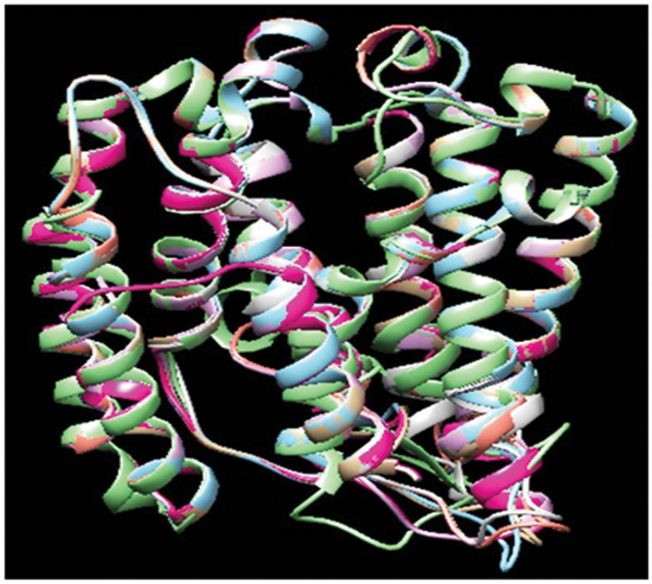

**Fig. 10.** The Superimposed Model.

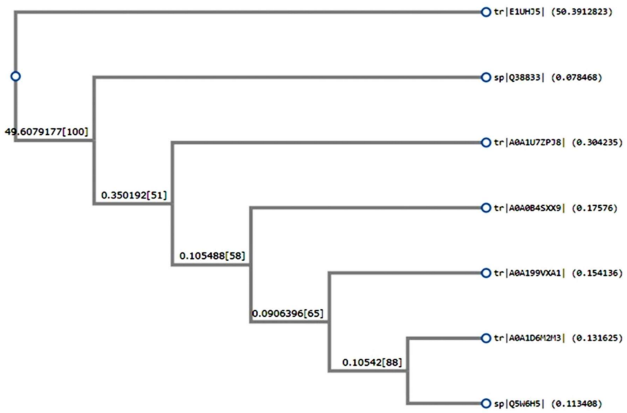


**Fig. 11.** Showing Results of Phylogenetic Tree Generated by CLUSTALW

### Multiple sequence alignment

The results of multiple sequence alignment using the CLUSTALW tool are shown in Figure 9. In addition, there has been a superimposed structure (Figure 10) generated based on seven different chlorophyll synthase enzymes.

### Phylogenetic tree analysis

The phylogenetic tree generated based on multiple sequence alignment through CLUSTALW, basically using the neighbor-joining (NJ) method, is given in Figure 11.

### Discussion

The final stage in chlorophyll biosynthesis is catalysed by chlorophyll synthase, which is the esterification of chlorophyllide with either geranylgeranyl diphosphate or phytyl diphosphate. Hence, this enzyme is the most important in the chlorophyll synthesis pathway. Consequently, whether a plant is monocot or eudicots, the plant contains this enzyme. In the present course of study, we aim at structural and evolutionary analyses of the enzyme present in different monocots and eudicots. For the study purpose, seven plants were chosen, namely *Arabidopsis thaliana, Zea mays, Oryza sativa, Camellia sinensis, Nelumbo nucifera, Musa balbisiana,* and *Ananas comosus.* Since the function of a protein or enzyme is dependent on its structure. Hence in-depth structural analysis is required for comparative study of the enzymes belonging to different plant species. Protein structures are categorized by the primary structure, secondary structure, tertiary structure, and quaternary structure. However, quaternary structural analysis was not performed since all enzymes contain only a single peptide chain. Instead, structural analysis of rest three hierarchical classes was performed.

### Primary structural Analysis:

First of all, their amino acid sequences were collected in FASTA format and subjected to Primary structure analysis. The primary structural components of seven different amino acid sequences of chlorophyll synthase enzyme belonging to seven different plant species have been represented in a tabulated manner, where values for various structural parameters including molecular weight, theoretical pI, number of both positively and negatively charged amino acids, extinction coefficients, instability index, aliphatic index and last but not the least grand average of hydropathicity (GRAVY) are given. From table 3, it can be well observed that the same protein, i.e., chlorophyll synthase from different plant species, shows a wide range of molecular weight (*Musa balbisiana* has the highest, i.e., 44635.74 gm. /mol and *Zea mays* has the lowest, i.e., 38886.26 gm. / mol.). Their theoretical pI also ranges between 7.62 to 9.89, a total number of positively charged residues is found to be highest in the case *Musa balbisiana,* i.e., 39, whereas the highest total number of negatively charged residues is shown by *Arabidopsis thaliana,* i.e., 27. In the case of instability index, if the value is found to be less than 40, then that protein is

declared as stable; here, only one protein shows an instability score of more than 40. Thus the chlorophyll synthase enzyme of *Musa balbisiana*(instability index- 51.80) is found to be unstable.The relative volume occupied by aliphatic side chains is defined as a protein's aliphatic index. It may be regarded as a positive factor for increasing the thermostability of globular proteins. From the results, the calculated aliphatic index of each of the seven proteins ranges between 104.63 to 116.30. Grand average of hydropathicity index (GRAVY) is used to represent the hydrophobicity value of a peptide, which calculates the sum of the hydropathy values of all the amino acids divided by the sequence length, according to the table chlorophyll synthase enzyme belonging to *Musa balbisiana*shows the maximum GRAVY score, i.e., 0.554, on the other hand, chlorophyll synthase enzyme belonging to *Arabidopsis thaliana*shows the minimum GRAVY score, i.e., 0.267. So from the entire primary structural component analysis, it is found that there are many structural differences present between monocots and eudicots, which make them distinctly significant from each other.

**Secondary structural Analysis:**

Secondary structural analysis(Figure 3) shows that the protein sequence of *Ananas comosus*(monocot)possesses the maximum amount of alpha-helices. In contrast, *Arabidopsis thaliana*(eudicot)bears a maximum amount of random coil and extended strand than other sequences. It is well recognized that most of the chlorophyll synthases of monocots have alpha-helices more than beta-pleated sheets. However, chlorophyll synthases of eudicots bear more random coil and extended strands comparable to others.

**3D Structure Prediction and Structural Quality Analysis:**

3D structures of chlorophyll synthase enzymes were not presentin Protein Data Bank; hence tertiary structures were predicted using the SWISS-MODEL server. The best model has been chosen based on multiple parameters like GMQE, QSQE scores, etc.

Model Structural Quality Analysis: The online server-generated 3D structures are undergone several quality analysis tests by various online tools, viz. PROCHECK, ERRAT, VERIFY-3D, VADAR, PROSAwere carried-out to get an overall idea about those protein structures.

Among all tools, PROCHECK is considered an important one because it analyzes Ramachandran Plot Statistics. According to the result, in the plot, Red region, deep yellow, light yellow, and white regions show the core, allowed, generously allowed, and not-allowed region, respectively. In the case of AT, 87.5% amino acids; in case of ZM, 88.5%; in case of OS, 89.6%; in case of CS, 87.4%; in case of NN, 88.1%; in case of MB, 89.8% and in case of AC, 87.6% amino acid residues are present in the most favored regions. The overall Ramachandran Plotstatistics (Table5)reveal the predicted models are of good quality and reliable.

Next, the overall quality factor of the models in the form of percentages was generated in a graphical manner using ERRAT. Proteins with higher scores indicate higher quality. The generally accepted range is >50 for a high-quality protein.Results originated via ERRAT give the overall quality factor of the model. The quality factor varies from 81.004% to 91.667%. Here, the overall quality factor determines the nature of the proteins.

The analysis is done by Verify-3D shows the result in the form of % of residues that have average 3d-1d score >= 2, whereas 3D quality analysis using VADAR showed the quality score range between 5 to 8 that indicate a good 3D profile of the predicted protein model. The model quality was further judged by the Z score obtained from the PROSA-Web server. ProSA-web result showed the overall z score of each protein. From the result table, the highest visible score is -3.17, which is of *the Zea mays* model and the lowest one is of *the Ananas comosus* model (- 4.38). That signifies that the predicted models are of good quality and comparable to X-RAY and NMR structures. Hence, they may be used for some advanced analysis.

**Total Energy Prediction:**

Next, the total energy determination of each protein was done via SPDBV and represented in a table form (Table 4).The total energy determined by Swiss-PDB Viewer Software gives

the total energy generated from the newly originated 3D protein structure, which allows the protein structure to be stable. The more negative the value is, the more energy stabilized the structure. From the data statistics, it is concluded, though the type of protein or, rather say, the enzyme is similar, still due to their origin of plant species, they show a varied range of total energy in the form KJ/mol. They start from -5082.891KJ/mol (*Camellia sinensis*; eudicot) to -8887.541KJ/mol (*Musa balbisiana*; monocot).

**Multiple Sequence Alignment and Phylogenetic Analysis:**

Amino acid sequences were collected in FASTA format and subjected to multiple sequence alignment and Phylogenetic analysis to understand their sequence similarity and phylogenetic evolution. From Figure 9, it has been observed that three different symbols indicate each amino acid's alignment with each other. E.g., "*" signifies perfect alignment; "." signifies a group of amino acids exhibiting weak similarity, whereas ":" signifies a group of amino acids showing strong similarity.Results highlighted that they have very high sequence similarities. Furthermore, superimposition of the 3D structures (Figure 10) of all the chlorophyll synthase reveals a high degree of structural similarity.

Next, we performed a Phylogenetic tree analysis. The phylogenetic tree (Figure 11) indicates the evolutionary tree. If the chosen matters are part of a single phylogenetic tree, they have a common ancestor. The meeting points of descendants are known as nodes. Each of these nodes is a taxonomic unit. From the phylogenetic tree analysis result, it has been observed that *Oryza sativa* and *Zea mays* together form a common clade. In contrast, *Musa balbisiana*is distantly related with*Oryza sativa* based on the evolutionary pathway.

## Conclusion

Chlorophyll synthase enzyme is an important player in chlorophyll biosynthesis pathway. Like chlorophyll biosynthesis pathway, this catalytic property of the enzyme class is also conserved in plant kingdom. Since, structure is the basis of function, the structure is also found to be very similar. Though their amino acid sequences are not completely identical, but a reasonable amount of similarities are present among them. They showed common ancestry in the phylogenetic tree. And quite naturally, they also have a good number of conserved regions.Structure prediction and in-depth analysis showed that, most of thechlorophyll synthases of monocots has alpha helices more than beta pleated sheets, although chlorophyll synthases of eudicots bears more random coil as well as extended strands comparable to othersHowever, the structural differences are less significant and hence, they are completely superimposed with one another as evident from aligned structures. The findings support the overall functional importance of the terminal enzyme of the chlorophyll synthesis pathway in evolutionary time scale.

## Acknowledgement

The authors are thankful to the Chancellor of Techno India University, West Bengal, for providing the necessary infrastructure.

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