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Functional prediction of *F3'5'H* in color alteration in cotton: an in-silico comparative analysis between cotton and viola

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Abstract

Anthocyanins are water-soluble pigments giving the purple, red and blue colors to various flowers and fruits. Anthocyanins are widespread amongst seed plants playing physiological roles such as to attract pollinators, seed dispersers and against UV–B protection. A key enzyme of this flavonoid pathway, Flavonoid 3', 5' hydoxylase,*F3'5'H* is involved in delphinidin production. Molecular level modifications in pigmentation pattern of cotton fiber, is an attractive and innovative area of research. In present study, comparison of *F3'5'H* in *Gossypium hirsutum* (Cotton) and *Viola* × *wittrockiana,* sequence alignment for homology, protein modeling along their structural validation and molecular docking analysis were computed. Docking studies were carried out using Autodock Vina and the ligands used were Naringenin and Quercetin. Analysis of results showed the best binding energy values of *Viola F3'5'H* i-e -8.9 & -9.9 with both ligands respectively. Based on these bioinformatics outcomes it was hypothesized that no matter the presence of this gene naturally in cotton, an over expression of *Viola F3'5'H* had a potency to alter pigmentation pattern in cotton fibers. This predictive screening method may prove to be significant for productive experimental studies and considered as a bypass for commercial scale research in terms of time and cost.

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Introduction

Cotton plays a vital role in economies of the countries worldwide. The ultimate product of cotton is fiber and textiles. Textile industry being the most important economic sector in Pakistan, contributes about 10 per cent to GDP and 55 per cent of the foreign exchange earnings of the country. Import of chemical dyes and pigments in Pakistan increased from $123 million in 2009-10 to $135 million in 2010-11, thus showing an increase of 4%. Import of organic chemicals in Pakistan increased from US $1.20 billion in 2006-07 to US $ 1.95 billion in 2010-11, thus showing an average increase of 13% per annum (http://www.ptj.com.pk/). Many chemicals used in the textile industry cause environmental and health problems. Therefore, need is to introduce eco-friendly textile product which on long term has no negative impact on our ecosystem.

Genus *Gossypium* is considered to be the vital source of cultivated cotton, oil and dietary protein for animals(Liu *et al*., 2009).Interest in adopting eco-friendly, wet-processing textile techniques has been increased in recent years, mainly because of improved awareness regarding environmental issues throughout the world (Ali *et al*., 2014).

*et alet al*Flavonoids are the pigments that impart color to most of the fruits, flowers and seeds anthocyanin pigmentation pathway has been studied extensively in *Zea mays, Arabidopsis thaliana, V. vinifera* and genes controlling each step have also been characterized (Boss *et al*., 1996; Sharma *et al*., 2012; Wang *et al*., 2011).

Two pairs of genes regulate Anthocyanin biosynthesis. One is of structural genes which encode for enzymes which catalyze the biochemical reactions for anthocyanin production. Whereas, the second set of genes encode transcription factors, which determine the spatial and temporal expression of anthocyanin accumulation structure genes. (Zhang *et al*., 2014)Member of cytochrome P450 family,*F3'5'H*, is a key enzyme in the synthesis of 3',5'- hydroxylated anthocyanidins (e.g delphinidin) (Holton, 2000). This enzyme induces two hydroxyl groups on the B ring of the flavonoid skeleton. Only plants that express the *F3'5'H* gene are capable of producing blue flowers, as these are dependent on 5'-hydroxylated anthocyanins. (Tanaka and Brugliera, 2008).

By genetic alterations it would be possible to achieve desired products of commercial value. (Fan *et al*., 2016) reported that expression of *LC* (leaf color) gene a bHLH (basic/helix-loophelix) anthocyanin regulator of maize, alone is sufficient to trigger the accumulation of anthocyanin in a variety of cell types including fiber cells in cotton. Therefore, it is hypothesized that by over expression of flavonoid structural genes, *F3'5'H* and *DFR* in cotton, there is a chance to alter pigmentation pattern in trichomes, which may in turn generate blue color.

The aim of current work was to inspect the possible existing relationship between experimental bioactivities of the ligands under study and the resulting docking scores. Thus, an in-silico comparative analysis between F3'5'H protein (*Gossypium* and *Viola*) has been carried out to elucidate difference in their respective protein structures and substrate utilization by protein-ligand docking. On the basis of obtained energy values from docking experiments, the functional efficacy of F3'5'H (*Gossypium & Viola*) could be predicted in terms of substrate utilization. Online search of accession numbers, Gen Bank: ACH56524.1 & Gen Bank: BAF93855.1 was done through NCBI website (http://www.ncbi.nlm.nih.gov) and sequence information was obtained.

Initiation codon was predicted using open reading frame finder (ORF) by NCBI and secondary structure of proteins was analyzed through PSIPRED. Hydrophobicity of both proteins was further evaluated by Prot Scale, however physiochemical properties were investigated using Protparam.

The present study will support the production of phenotypic alterations in cotton fibers In-vitro. Moreover, postulated role of *F3'5'H* (*Viola*) in altering

anthocyanidin pathway towards delphinine production in many of the ornamental flower plants i.e .transgenic blue rose and blue *Chrysanthemums* has been documented previously*(Katsumoto et al., 2007)*(Katsumoto *et al.*,2007; Noda *et al.*, 2013).

Materials and methods

Computational approaches were applied to suggest potential research procedures for exploration of molecular mechanisms up to greater extent. Protein-ligand docking with estimation of binding affinity of the complex is an important part of synthetic and structural biology. For this assessment, following comparison in F3'5'H proteins of *Gossypium hirsutum and Viola × wittrockiana* was conducted.

Protein Sequence alignment and primary analysis

Initially, the protein sequences of *Gossypium*, *GhF3'5H* (accession no. ACH56524.1) and *Viola × wittrockiana* (accession no.CEO43477.1) were retrieved from NCBI gene bank and aligned to check sequence homology by CLC Genomics Workbench 8.The hydrophobic portion of F3'5'H proteins along with generated hydropathy plots was obtained using EXPASY ProtScale online tool (http:// web.expasy.org/protscale/).The said tool showed graphical representation of hydrophobic values of all amino acids present in the sequence.

The Prot Param Server in expasy platform (http://web.expasy.org/protparam) was further used to compute the physiochemical characteristics of the targeted protein sequences by using the protein sequence in FASTA format as the input data type. These properties included isoelectric point (pI), aliphatic index (AI), extinction coefficients, GRAVY (grand average of hydropathy) and molecular weight.

3D Modelling, evaluation and docking of receptor molecules

For computing the secondary structural characteristics of *F3'5'H* amino acid sequences (*Gossypium hirsutum and Viola × wittrockiana*) PSIPRED server of UCL Department of Computer Science (http://bioinf.cs.ucl.ac.uk/psipred/), was used.

3D model construction

Since the required F3'5'H protein (*Gossypium hirsutum*and *Viola × wittrockiana*) structures were not available on PDB, so their 3D models were generated through I-TASSER online server (http://zhanglab.ccmb.med.umich.edu/I- TASSER/). This tool produced tertiary structure along with their confidence score (C-Score). Predicted models were viewed in Ras Mol and undergone docking experiments.

Model validation

ProSA-Protein Structure Analysis program is a sensitive method in distinguishing overall correct and incorrect folds of proteins. It works statistically by analyzing all available protein structures for the potential errors. This web server by evaluating energy profile along total energy deviation verified the protein structures in terms of Z score, which represented the overall protein model quality (Wiederstein and Sippl, 2007). Greater the negative values, more stable would be the protein structure. Furthermore, the validation studies of the generated models of *F3'5'H* was carried out using PROCHECK program.

Docking protocol

Auto Dock/Vina with its default parameters was employed for docking, using protein-ligand information along with grid box properties in the configuration file. This software provides rapid and accurate binding mode predictions than any other docking software. It is based on a sophisticated optimization algorithm that uses a gradient optimization method in order to calculate the binding energy of the receptor-ligand complex. These docking experiments were conducted in order to explore the substrate utility efficacy of receptor proteins F3'5'H with its respective ligands, Naringenin and Quercetin. The ligands, Naringenin (CID: 932) and Quercetin (CID: 5280343) were downloaded from pub Chem database in 3D format and converted to pdb file,

because the auto dock software could only recognize files in pdb format. The Pdbqt files of the receptor and ligand molecules were generated by using the Auto dock tools software downloaded from MGLm tools (http://mgltools.scripps.edu/downloads). In order to generate receptor pdbqt files, water molecules were removed, hydrogen atoms and kollman charges were added followed by preparation of grid box to cover whole molecule in order to make it ready for ligand binding. Similarly, pdbqt file for ligand was prepared and root was detected by going to torsion tree. For preparation of the grid box, size was set as 30 × 30 × 30 xyz points with spacing (grid) of 1 Å. Broyden-Fletcher-Goldfarb-Shanno algorithm was used for docking calculations. The ligand binding orientation and conformations (generally known as posing) was predicted by Search algorithms (Rauf *et al.*, 2015; Seeliger and de Groot, 2010). The obtained output files of docking experiments were opened in Pymol. After visualization of the receptor-ligand complex, the final interpretation was made.

Results

Comparison of Sequence homology &hydrophobic regions

The two aligned sequences showed 77 % homology (Fig. 1) and 33% dissimilarity. This is a significant difference in amino acid sequences as even a single amino acid substitution could affect the function of aparticular gene. Using, Hphob. / Kyte & Doolittle scale, the graphs were generated which showed the Tran membrane regions of *Viola× wittrockiana* and *Gossypium hirsutum* (Fig. 2a & b). The plots predicted position of amino acids at 'X' axis and hydrophobic score along 'Y' axis. As per *ViolaF3'5'H* results, the hydrophobicity values range from3.567 (position 13 aa) to -2.6 (position 361 aa) while in *GhF3'5'H* similar region exists from 2.88 (position 17 aa) to -2.72 (position 345 aa) which showed that amino acids in these regions were embedded in phospholipid bilayer. These trans membrane proteins function as gateways to allow the transport of specific substances across the biological membranes.

Table 1. ProtParam tool analysis of Viola × wittrockiana & *Gossypium hirsutum*.

Accession number	No. of amino acids	MW	pI	Asp + Glu	Arg+ Lys	Aliphatic index (AI)	Instability index (II)	Grand average of hydropathicity (GRAVY)
CEO43477.1	506	56056.5	8.92	52	59	98.34	33.86	0.019
ACH56524.1	510	57371.9	9.14	54	63	91.61	38.69	0.137

Table 2. Binding energies of compounds interaction computed by Auto Dock/vina.

Protein	Ligand	Binding energy (KJ/mol)	No.of hydrogen bonds	Bonded amino acids
Gossypium hirsutum	Naringenin	-7.7	1	THR 370
	Quercetin	-7.5	2	ARG 439, ARG 445
Viola× wittrockiana	Naringenin	-8.9	5	TRP 125, THR 366, ASN 369, PHE 436, ARG 441
	Quercetin	-9.9	9	THR 366, ASN 369, PHE 436, ARG 441

For secondary structure prediction of proteins PSIPRED server was used. The output data revealed that viola secondary structure contained alpha helix 47.04%, random coils 39.92% as well as extended strands 13.04% (Fig. 3) while *Gossypium* had most frequent alpha helix and random coils which were found to be 42.75% followed by 13.04% extended strands (Fig. 4).

Prot Param tool revealed the physiochemical properties of amino acid sequences (*Viola × wittrockiana* & *Gossypium hirsutum)* as shown in Table 1.

Stability index values for both proteins showed their stable nature. Half-life of a protein is defined as a function of the nature of its amino-terminal residue.

The half-life is a prediction of half time length of a protein to vanish after its synthesis in the cell. The N-terminal of the both amino acid sequences had M (Met).Estimated half-life for both proteins in mammalian reticulocytes was 30h, while in yeast and *Escherichia coli* were more than 20 and 10h, respectively.

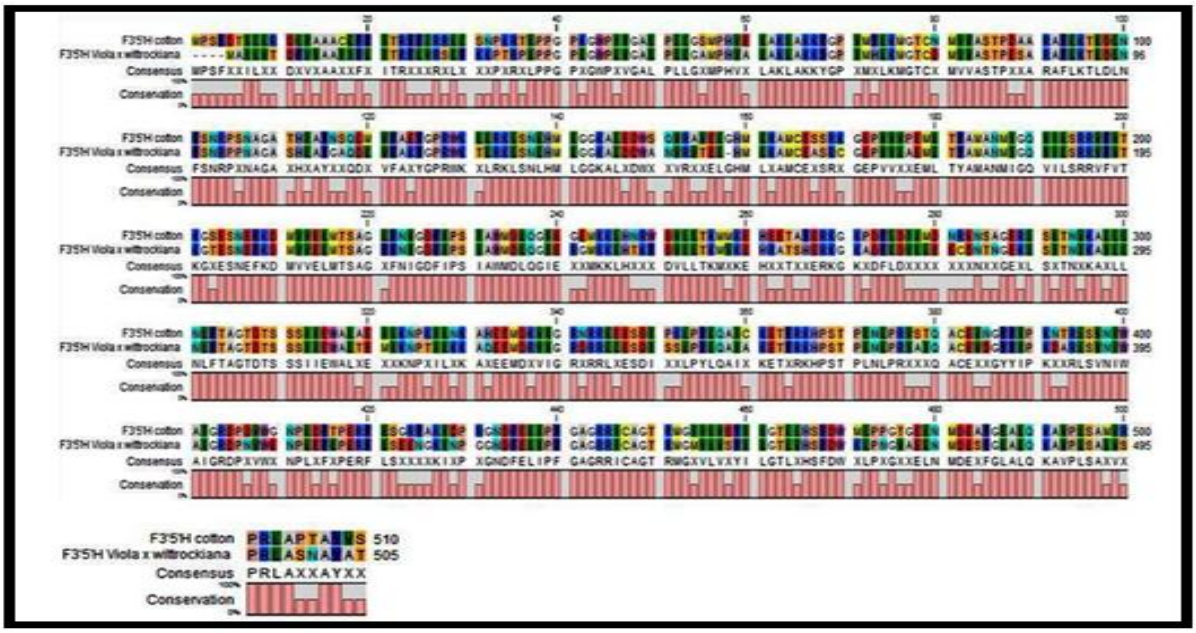

Fig. 1. Consensus amino acid sequences alignment of F3'5'H from *Gossypium hirsutum* and *Viola* × *wittrockiana* by CLC Genomics Workbench 8.The colored bars at the bottom represent the conservation Percentage.

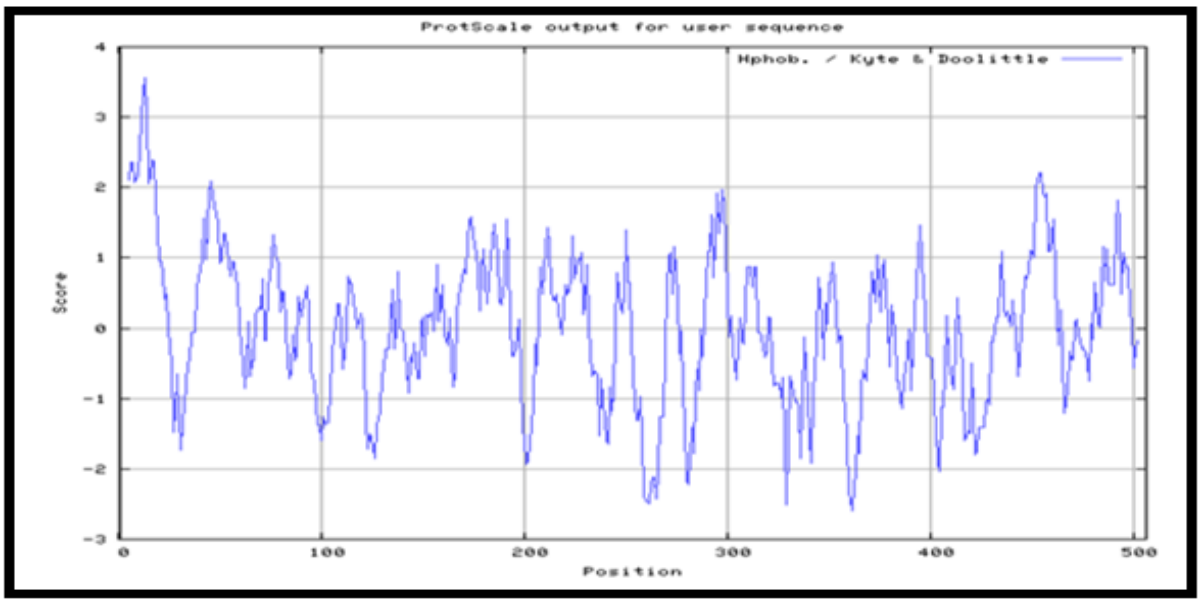

Fig. 2A. Hydrophobic graph prediction of *Viola* × *wittrockiana* generated by Prot Scale online tool. Portion of graph below threshold line showed hydrophilic & above area defined the hydrophobic region.

Evaluation and validation of refined models

Protein models of *Viola* × *wittrockiana* and *Gossypium hirsutum* made by I-TASSER (Fig. 5 a,b). Validations of produced 3D models were further analyzed by Pro SA-web service, ERRAT and VERIFY 3D programs.

Pro SA server

Pro SA service calculated the Z score of the proteins under study and was displaced in the plot with a dark black dot. These graphs contained the Z-score of all experimentally determined protein chains in given PDB file. Groups of structures from different sources (X-ray, NMR) were distinguished by different colors which further used to check whether the Z-score of the input structure was within the range of scores found typically for native proteins of similar size.

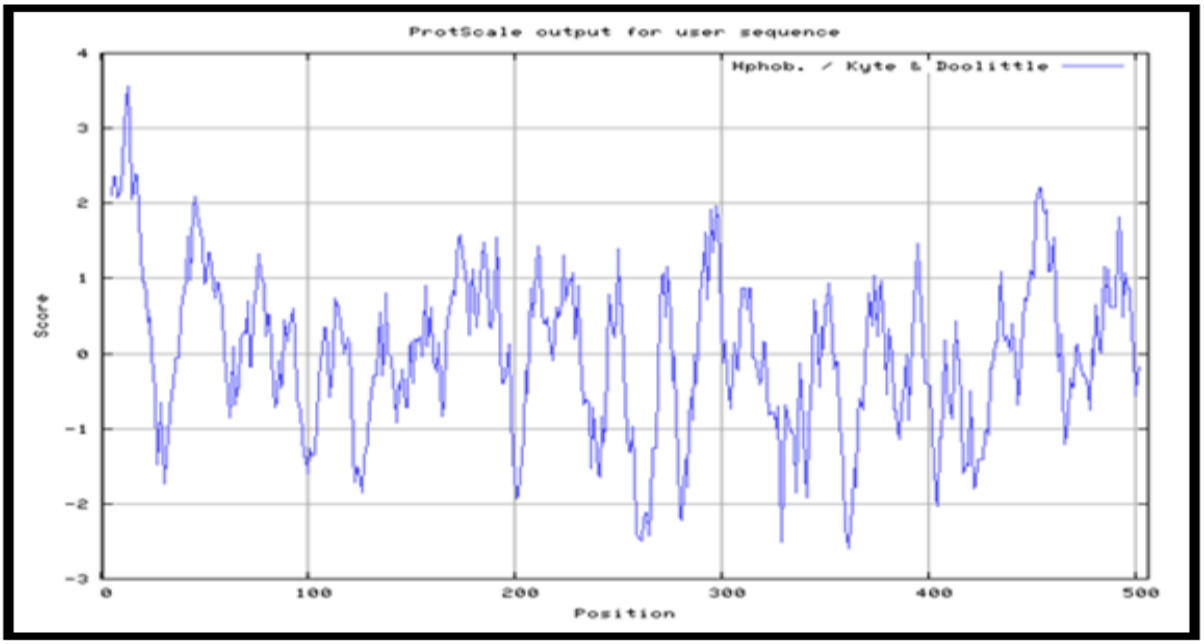

Fig. 2B. Hydrophobic graph prediction of *Gossypium hirsutum* generated by Prot Scale online tool. Graph showed both hydrophilic & hydrophobic regions in respective protein sequence.

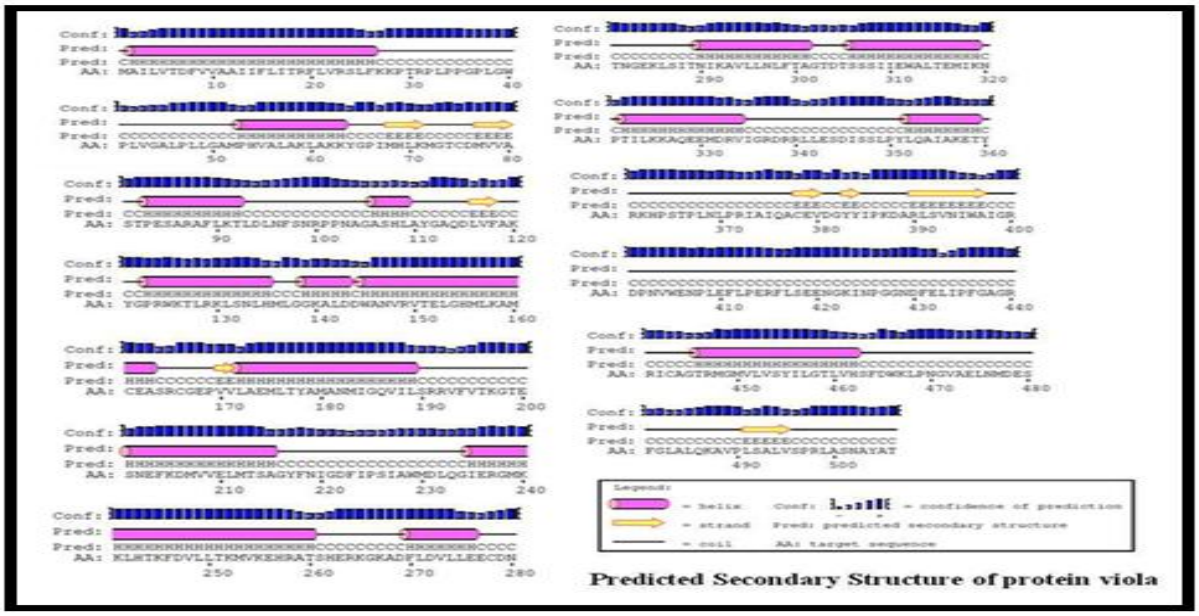

Fig. 3. Predicted Secondary structure for *viola* × *wittrockiana* by PSIPRED online server. Pink rods: α-helices, yellow arrows: β-strands, black lines: coils. Blue bars on the top indicated confidence of prediction.

The Z score value of the obtained model was −7.39for *Viola*&- 4.16 for *Gossypium* respectively (Fig. 6 & 7), which was within the acceptable range −10 to 10**.** Higher the negative Z-score values, higher would be the quality of protein model.

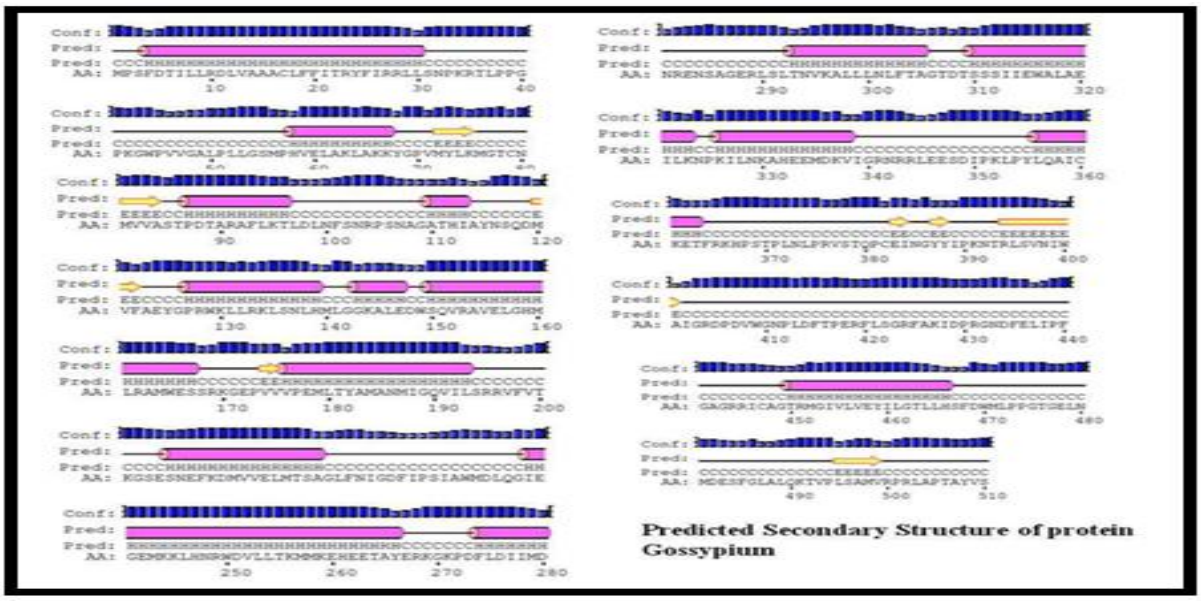

Fig. 4. Predicted Secondary structure for *Gossypium hirsutum* by PSIPRED online server. Pink rods: α-helices, yellow arrows: β-strands, black lines: coils. Blue bars on the top showed confidence of prediction.

The second plot (Fig. 7) shows the local model quality by plotting energies as a function of amino acid sequence position. Basically, folding energy of the protein showed minimum value as it accounts for stability and nativity of the molecule. Positive values correspond to problematic or erroneous parts of the input structure.

The dark thicker green line represents the energies of model which indicate their stability. Quality assessment of the 3D structures by Pro SA revealed their stability. Furthermore, other structure verification servers such as Verify 3D and Errat were used for the validation of refine modeled structures.

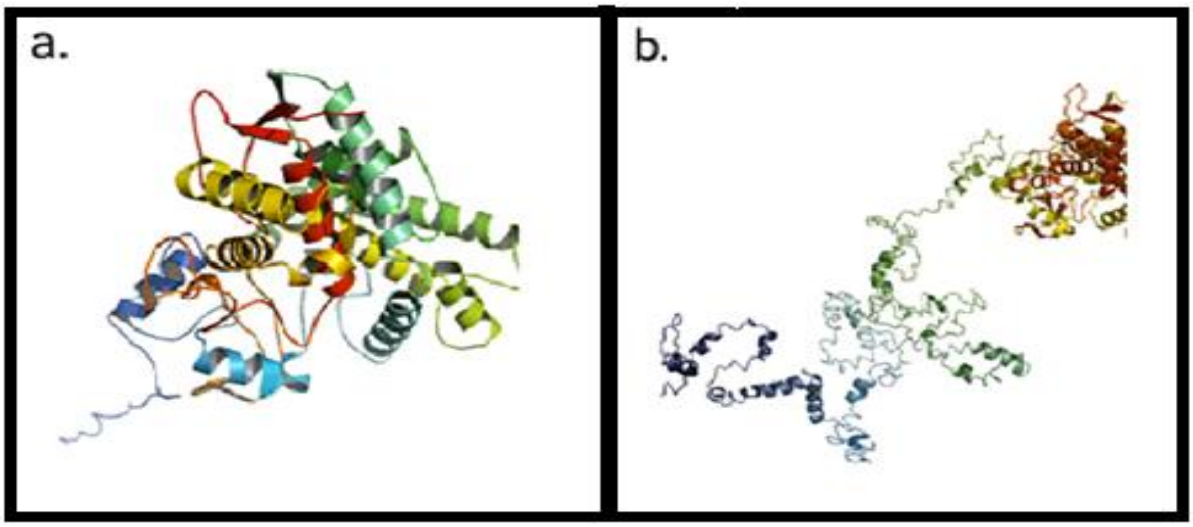

Fig. 5. A.3D models of *Viola* × *wittrockiana* predicted by I-TASSER, B.3D models of *Gossypium hirsutum* predicted by I-TASSER.

Errat

Errat is a protein structure verification algorithm that is particularly suitable for estimating the progress crystallographic model building and refinement. This program specifically worked by investigating the statistics of non-bonded interactions among different atom types. ERRAT analysis revealed the overall quality factor of 88.75 for *Viola* as well as 86.45 for

Gossypium model respectively.

This obtained data implied that overall quality factor of both protein models is very good. The plots generated by Verify-3D and Errat for receptor molecules were displayed in Fig. 8A, B and Fig. 9A, B.

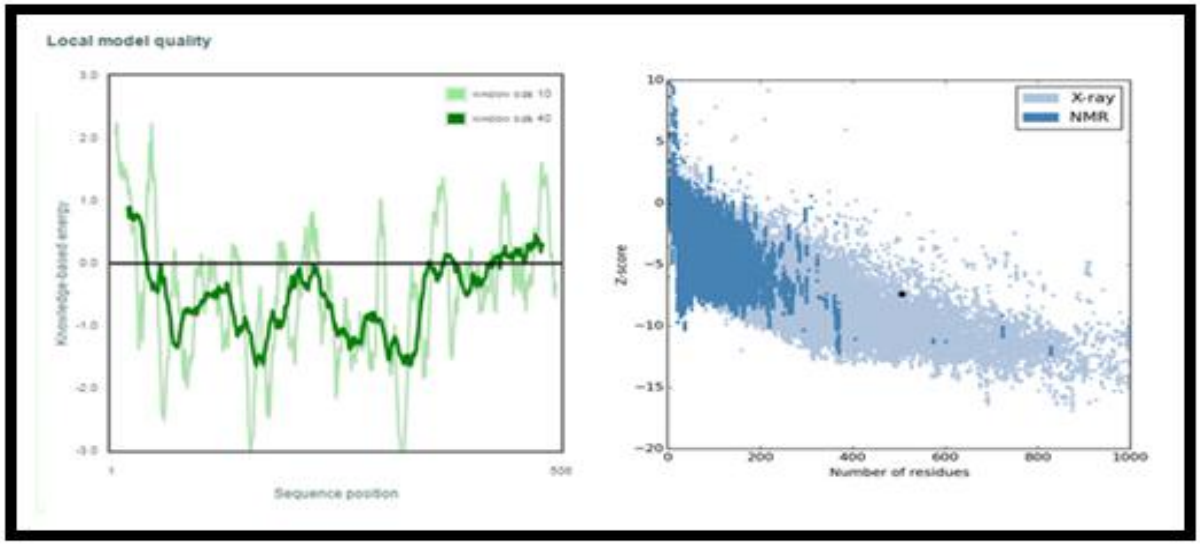

Fig. 6. ProSA overall and local quality plots for *Viola* × *wittrockiana* with Z-score of -7.39. Theblack dot in left hand graph represents Z score of the *Viola* model.

Verify 3D

It is used to validate the refine models. The 3D model of a protein was compared to its amino acid sequence by considering a 3D profile calculated from the atomic coordinates of the structures of the correct proteins (Eisenberg *et al.*, 1997). Otherwise an incorrectly modeled structure can be identified by examining the profile score in a moving-window scan. Thus, the correctness of a protein model can be verified by its 3D profile, regardless whether the model has been retrieved by nuclear magnetic resonance (NMR), X-ray, or any other computational procedures.

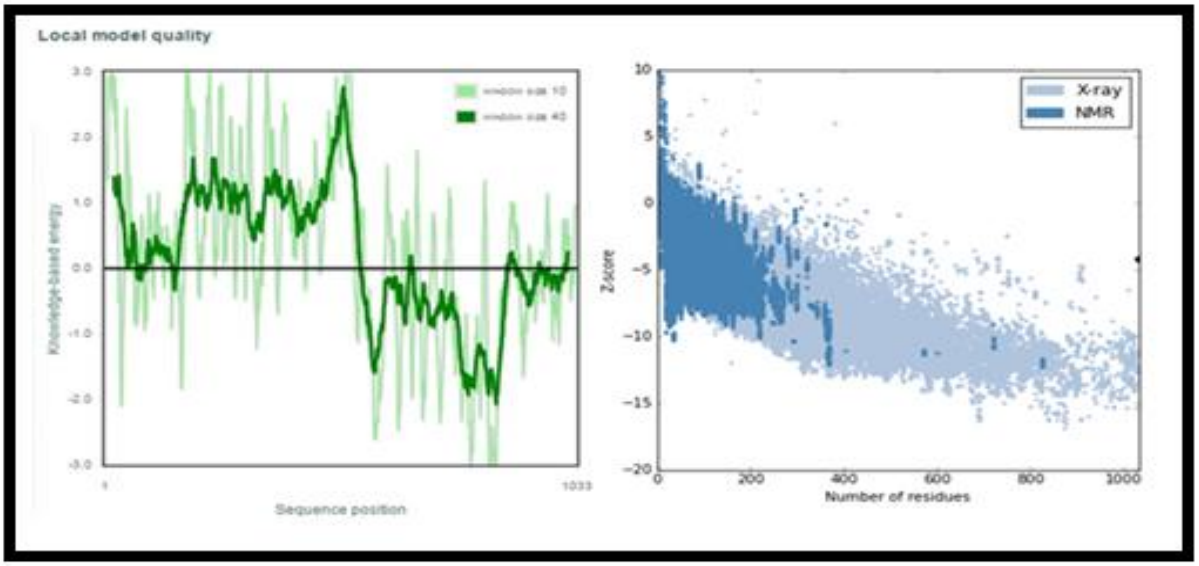

Fig. 7. Pro SA overall and local quality plots for *Gossypium hirsutum*. The black dot showed all protein chains in PDB determined by X-ray crystallography (light blue) or NMR spectroscopy (dark blue) with respect to their length.

Verify 3D allotted a 3D-1D score of >0.2 for the both modeled proteins. Evaluation of the model by Verify3D therefore revealed that 76.4 % of residues had an average score>0.2 in case of *Gossypium* whereas 80.04% of the residues attained an averaged 3Dscore >0.2 in *Viola* (Fig. 8A, B). These computed results validated the three dimensional protein structure.

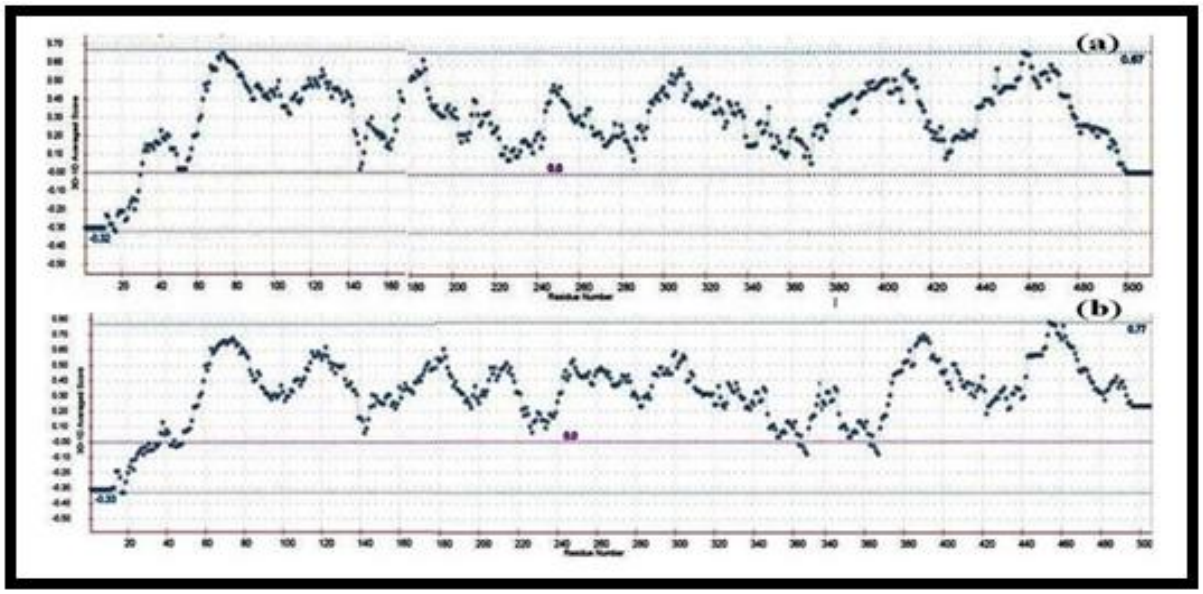

Fig. 8. Verify3D profile for models *Viola* × *wittrockiana* (a) and *Gossypium hirsutum* (b).Scores over threshold line indicate a high quality model.

Protein-Ligand docking analysis

The docking studies indicated that *F3'5'H* enzyme from Viola species showed better binding energies with both substrates (i.e, Naringenin and Quercetin) in comparison to *Gossypium* (Table 2).

The binding energy with Naringenin was found to be -8.9 kcal/mol and with Quercetin, -9.9 kcal/mol. However, *GhF3'5'H* displayed binding energies as -7.5kcal/mol & -7.7 kcal/mol with Naringenin as well as Quercetin respectively. On the basis of binding energies evaluation, it was demonstrated that in either case (*Gossypium hirsutum* or *Viola* × *wittrockiana*) *F3'5'H*consumed quercetin more efficiently as substrate as compared to Naringenin. The docking poses were dependable to their docking scores. The docked poses having least binding energy showed the highest binding affinity with its respective substrate and hence considered the best docked conformation. The generated docking poses could be directly loaded in Ras MOL. Moreover, amino acids found in binding sites were summarized in a table 2 (Fig.10).

Within *GhF3'5'H,* protein residues such as THR 370, ARG 439 and ARG 445 interacted with Naringenin and Quercetin. From Viola F3'5'H protein, most of the docked results showed interacting physiognomies of TRP 125, THR 366, ASN 369, PHE 436, ARG 441, THR 366, ASN 369, PHE 436 and ARG 441 amongst all other residues.

These amino acids directly contribute in the catalytic action of this enzyme. Helping it form hydrogen bonds, ionic and hydrophobic interactions with the key residues of the binding pocket of substrate thus stabilizing the structure of target receptors.

From all the generated data by molecular docking it was shown that *F3'5'H* from source Viola more efficiently consumes substrates, Naringenin and Quercetin than *GhF3'5'H,* which depicts its comparatively proficient ability to generate blue pigment producing protein-delphinidin, hence giving blue shade to the plant.

Discussion

Studied parameters and approaches

Primary sequence analysis, using expasy tools such as Prot Param and Prot Scale showed the stability and basic nature of the proteins. Similar kind of analysis has been performed formerly for various substrates and proteins (Shen *et al.*, 2015).

Tertiary structural details of the proteins were significant in providing insight to their molecular functions**.** The Z-score of the 3D-models were calculated as -7.39 &- 4.16, which fall in the acceptable range of -10 to 10. It has been reported that Z score corresponds not only to the overall quality but also calculates the deviation of the total energy of a protein structure. In general, Z score is dependent on the protein length (Yadav *et al.*, 2012).Minimum value of folding energy of a protein accounts for the nativity and stability of the molecules.

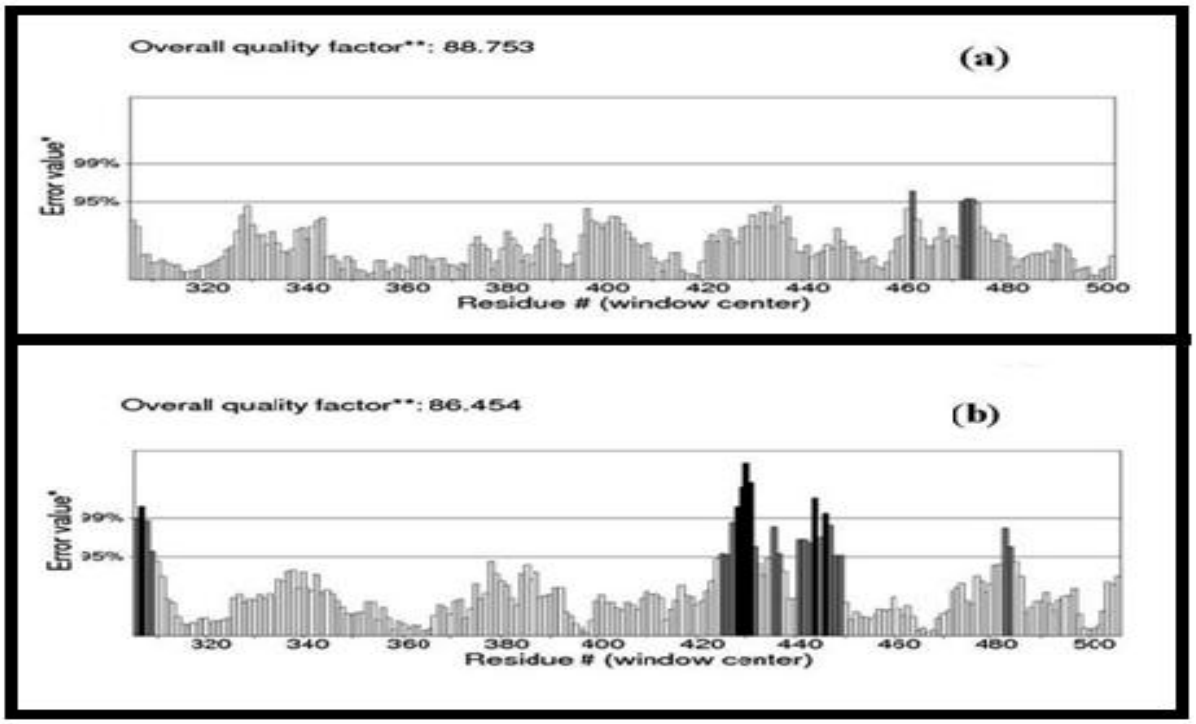

Fig. 9. Errat plots of protein models (a) *Viola* × *wittrockiana* and (b) *Gossypium hirsutum*. Overall quality factor above 80 showed a good stable model.

In background of the above analysis, intermolecular energy of two compounds Naringenin and Quercetin (–7.7: -7.5 Kcal/mol and -8.9: –9.90 Kcal/mol) along the docked poses created by Auto Dock Vina produced the best results.

This ligand–enzyme complex was stabilized by hydrophobic interactions and hydrogen bonds. Present results were in accordance with Steiner and Koellner, (2001) who demonstrated that greater the number of hydrogen bonds, stable would be protein-ligand complex. A similar study by (Gupta *et al.*, 2013)explained that two novel compounds (Chem Bank ID 2110359 and 3075417) were found to be more potent inhibitors of neuraminidase than control drugs as oseltamivir and zanamivir in the background of their robust binding energies. Even a single amino acid alteration in active site can be responsible for working different functionality of proteins. Idea of development of broad-ranged Vip3Aa-Cry1Ac fusion protein against the pests of cotton crop particularly *Lepidopteran* was showed using molecular docking approach i.e. Z- Dock server. Moreover, the authenticity of proposed hypothesis was checked by transforming this fusion gene into cotton embryos (Ahmad *et al.*, 2015).

Using docking software i-e auto dock 4.0, the ant diabetic activity of *Helicteres isora* was screened out and the most potent ant diabetic constituent was identified (Aleykutty, 2012).

Analysis

Functionally active P450 enzymes required coupling with other genes i-e *DFR* that could act as an electron donor in order to generate naturally colored phenolic pigments. In the flavonoid pathway, same gene showed variation in substrate preference or specificity in different plant species. For example, *DFR* genes highly vary in substrate specificity. Some *DFR*s had the ability to consume DHQ in one plant species while others utilized DHM as substrates to produce red or blue flowers. Further, it was found that deletion of proline rich region near position 12 resulted in loss of *DFR* activity and eventually DHK reduction. Therefore, it was an important consideration to determine over expression of which particular gene would efficiently consume substrate in that specific crop.

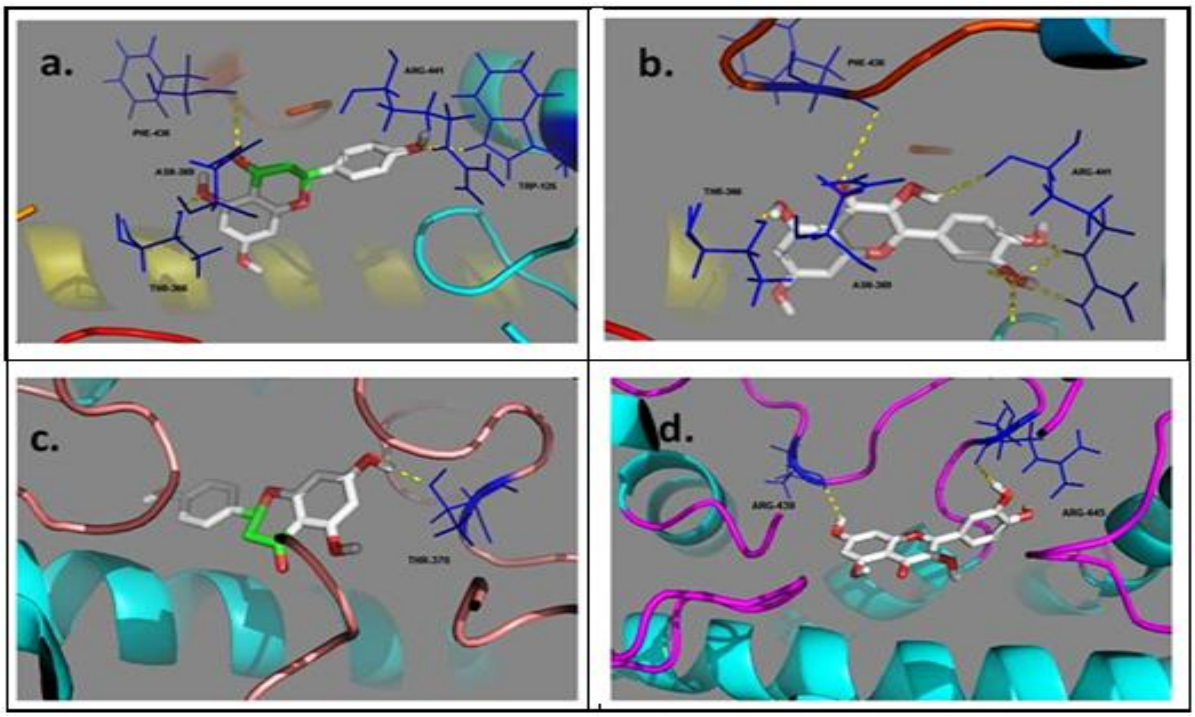

Fig. 10. Docking analysis of *Viola* × *wittrockiana* and *Gossypium hirsutum* with Naringenin & Quercetin. *ViolaF3'5'H* docked with (a) Naringenin & (b) Quercetin. *Gossypium F3'5'H* docked with (c) Naringenin & (d) Quercet.

The choice of enzyme which could actively devourits respective substrate to get desired results was an actual enigma (Ahad *et al.*, 2015).

Current comparison by Auto dock Vina of *F3'5'H*protein from two different sources clearly showed that *ViolaF3'5'H*protein had greater potency to utilize its respective substrate rather than *GhF3'5'H*. Hence there exited a brighter prospect to alter the pigmentation pattern of cotton fiber.

This hypothesis is supported by (Katsumoto *et al.*, 2007; Noda *et al.*, 2013) who genetically engineered *Chrysanthemums* and achieved bluer color on delphinidin accumulation on the overexpression of *CampanulaF3'5'H*. Similarly blue roses were generated by the transformation of Viola *F3'5'H*gene along with down regulation of endogenous *DFR* and overexpression of *Iris DFR* (Katsumoto *et al.*, 2007). Presented results were in accordance with our previous work (Ahad *et al.*, 2015) where we have introduced an in-silco approach to alter pigmentation

pattern and proposed that ecofriendly colored cotton can be produced.

Outcome of study

The study has successfully predicted the consistent computational 3-D models for *F3'5'H* protein, which is the highly targeted protein in bioengineering of flavonoid pathway. This is the most targeted protein used in color modifications at molecular level. Pro SA service, ERRAT Verify 3D servers have further validated the developed *F3'5'H*models. Molecular docking studies were carried to screen out the binding affinities between receptor and ligand molecules. Obtained robust binding energy along strong hydrogen bond interactions in docking experiments supported the novel idea that over expression of *F3'5'H*gene from source *Viola* to *Gossypium* species had a chance to alter pigmentation pattern in cotton fibers on the basis of its ability to consume substrate more efficiently. Thus, the deposition of these water soluble pigments has a potential to generate different colors. Further verification through in-vitro experiments can testify the proposed idea and lead to successful production of eco-friendly colored cotton or other commercial uses in accord with needs.

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