Multi-faceted study of EDS1 gene in *Citrus sinensis* (sweet orange); a bioinformatics study

Sauood Shaheer Siddiqui[1], Irfan Mughal[2], Mudassar Fareed Awan[1], Sajed Ali[1], Muhammad Farhan Sarwar[1*]

[1, 1*]Department of Biotechnology, Knowledge Unit of Science, University of Management and Technology (UMT) Sialkot, Pakistan
[2] Department of Life and Environmental Sciences, Deakin University, Australia

ABSTRACT

The EDS1 gene contributes to numerous plant defence and protection mechanisms and confers resistance to biotic stresses brought on by pathogens. It encodes an alpha/beta hydrolase with a conserved domain that facilitates plant defence. In this research, a genome-wide investigation and the identification of EDS1 genes in Citrus sinensis (sweet orange) were carried out. In sweet orange, we found three distinct orthologs of alpha/beta hydrolase through phylogenetic and conserved domain analysis. Sub-cellular localization investigations revealed that this protein was distributed throughout the cell, particularly in the nucleus. In this regard, chromosomal mapping and gene duplication analyses were also carried out. Additionally, the EDS1 gene's corresponding protein's amino acid sequence was incorporated in order to perform the three-dimensional (3D) structure prediction. Its enzymatic nature was further examined using active site prediction. All of the predicted protein structures contained various active sites, which we discovered. Future research might use this findings as a basis for continuing to investigate the EDS1 gene from a variety of aspects in order to fully understand the critical roles, it plays in *Citrus sinensis*.

Keywords: Alpha/beta hydrolase superfamily, Biotic stresses, *Citrus sinensis*, Defense response, Genome wide analysis

INTRODUCTION

EDS1 (enhanced disease susceptibility) gene, a mediator of plant stress and disease response, is involved in mechanism of plant's defense in response to infection with certain pathogen. This fact is evident of its importance as its role in the functionality of R gene of *Arabidopsis thaliana*. It has structural similarity to lipase family of eukaryotes and involvement in hydrolysis of lipid-related molecules may be crucial to it its protective role [1]. EDS1 gene also confers resistance to plant disease by triggering pathogenesis-related protein expression [2]. EDS1 also interacts with other factors such as BZR1, a regulatory element in brassinosteroid signalling and consequently plays its part in basal defense mechanism [3]. Inhibition or repression of EDS1 may result in poor defense response as evident by the fact that suppressing its transcription in *Arabidopsis thaliana* resulted in poor accumulation of salicylic acid, in response to plant pathogen [4]. Similarly, in

other plant species such as tomato, it is responsible in defense mechanism against certain pathogens such as viruses, bacteria and fungi [5].

Citrus fruits are of considerable importance not only for their nutritional importance but also of their waste materials such as their peel can be used for obtaining various useful products which may include carotenoids, flavonoids, dietary fibers, many oils and ascorbic acid. Its peel may also contain considerable amount of sugar products which could be used in fermentation for the production of bioethanol [6-8] and also methane can by yielded from the waste of oranges peel [9]. Furthermore, many immunity related benefits are also associated with consumption of citrus fruits as they are source of vitamin C (ascorbic acid) and also boost antioxidant activity [10]. Similarly, orange juice also improves immunity by reducing inflammation as it is a good source of folate and other bioactive compounds [10]. Due to such important factors and diverse applications of citrus fruits for various purposes, they have to be studied from every perspective including molecular and cellular elementary characteristics.

Till date genome wide analysis and studies of different transcription factors and proteins of *Citrus sinensis* has been done such as genome wide annotation of *Alkb* gene family, NBS-LRR (nucleotide-binding site-leucine-rich repeat gene) gene family, major intrinsic proteins, SBP-box gene family and TALE transcription factors [11-15]. Very less number of studies have been reported on genome wide analysis of EDS1. Only, the genome wide analysis of EDS1 has been reported in brinjal plant [16] but no work has been reported till date regarding genome wide studies of enhanced disease susceptibility in citrus family.

In this study we have worked on this particular aspect i.e., the genome wide study on EDS1 gene in one of species of citrus family i.e., *Citrus sinensis* (sweet orange) through different in-silico approaches including phylogeny study, exon and intron analysis, conserved domain and motif analysis, subcellular localization, chromosomal mapping, among other important attributes. This study may help other scientists to evaluate its role in protection against biotic stress and may prove to be beneficial in conferring resistance against different types of plant pathogens. It is evident from previous study that EDS1 is an important mediator in plants systemic defense against pathogens so evaluating its structural and functional role may prove to be beneficial in enhancing plants protection mechanism against certain infections and can help improve production of fruit. Nevertheless, more studies will be needed to fully comprehend and utilize this protein for maximum benefits.

MATERIALS AND METHODS

Data retrieval:

The nucleotide sequence of EDS1 gene of *Arabidopsis thaliana* was obtained from NCBI under accession number as NP_001030829.1. The domain search was done through InterPro (https://www.ebi.ac.uk/interpro/search/sequence/) [17]. BLAST analysis of of Alpha/beta

hydrolase domain was done with threshold value of 1e-10 through CPBD: Citrus Pan-genome to Breeding Database (http://citrus.hzau.edu.cn/index.php) [18]. Five types of sequences were retrieved including peptide, promoter, coding sequence, cDNA and genomic from the generated results. Moreover, the gff3 files were also downloaded from same database. The selected sequence was analyzed by InterPro to check the presence of alpha/beta hydrolase.

Phylogenetic Analysis:

For phylogenetic analysis, the sequences of EDS1 gene were retrieved from phytozome v.13 (https://phytozome-next.jgi.doe.gov/) [19]. The plants selected in this regard, were *Arabidopsis thaliana*, *Oryza sativa* and *Zea mays*. MEGA 11 was incorporated for this purpose [20]. ClustalW method was opted for alignment while, neighbor-joining (NJ) with a bootstrap value of 1000 for tree construction which was further annotated with itol web server (https://itol.embl.de/) [21].

Exon/Intron Structure:

Exon/intron structure was depicted through GSDS 2.0 gene structure display server (http://gsds.gao-lab.org/) [22].

Domain analysis:

For analyzing domains in our sequences NCBI's conserved domain search feature (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was employed [23]. Alpha/beta hydrolase super family was found in all uploaded peptide sequences of *Citrus sinensis*.

Motif analysis:

Motif analysis was done through MEME suit (https://meme-suite.org/meme/)[24]. Total of 10 motifs were found for each sequence through this online database.

Subcellular location:

For subcellular localization prediction an online tool, WoLF PSORT (https://wolfpsort.hgc.jp/) was used. It predicted the subcellular location of given peptide sequences [25]. The details were summarized in the form of table. Moreover, a heat map through tbtools was also produced for better visualization [26].

Physio-chemical properties:

For physiochemical properties analysis, Expasy ProtParam tool [28] was utilized (https://web.expasy.org/protparam/).

Chromosomal mapping:

Chromosomal mapping was done through an online web tool m2gc v2.1 (http://mg2c.iask.in/mg2c_v2.1/) [29].

Synonymous and nonsynonymous substitution:

For the calculation of synonymous and non-synonymous substitution we made use of gff3 file downloaded from citrus genome database and with the help of tbtools calculated ka and ks value and found duplication events and time (for duplication we used λ=1.5*10^-8).

3D models and binding sites prediction:

The prediction of 3d structures was done by utilizing an online tool Protein Homology/analogY Recognition Engine V 2.0 Phyre2 (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) [30]. While, for the prediction of binding sites, the pdb files from this tool were uploaded on CASTp 3.0 (http://sts.bioe.uic.edu/castp/index.html?4jii) [31].

RESULTS

Evolutionary analysis:

Phylogenetic tree of alpha/beta hydrolase is shown in figure 1. This tree was constructed with neighbour joining method and with bootstrap value of 1000. The relevant species that were subjected to construct the tree were *Oryza sativa*, *Zea mays*, *Arabidopsis thaliana* and *Citrus sinensis*. Four main clades were identified which are shown in the figure. These clades are indicated with different colors and sequences of sweet orang are distinguished by red dot alongside. The results suggest that sweet orange showed the closet similarity with *A. thaliana* which indicated that this gene may share same evolutionary background in both of these species.

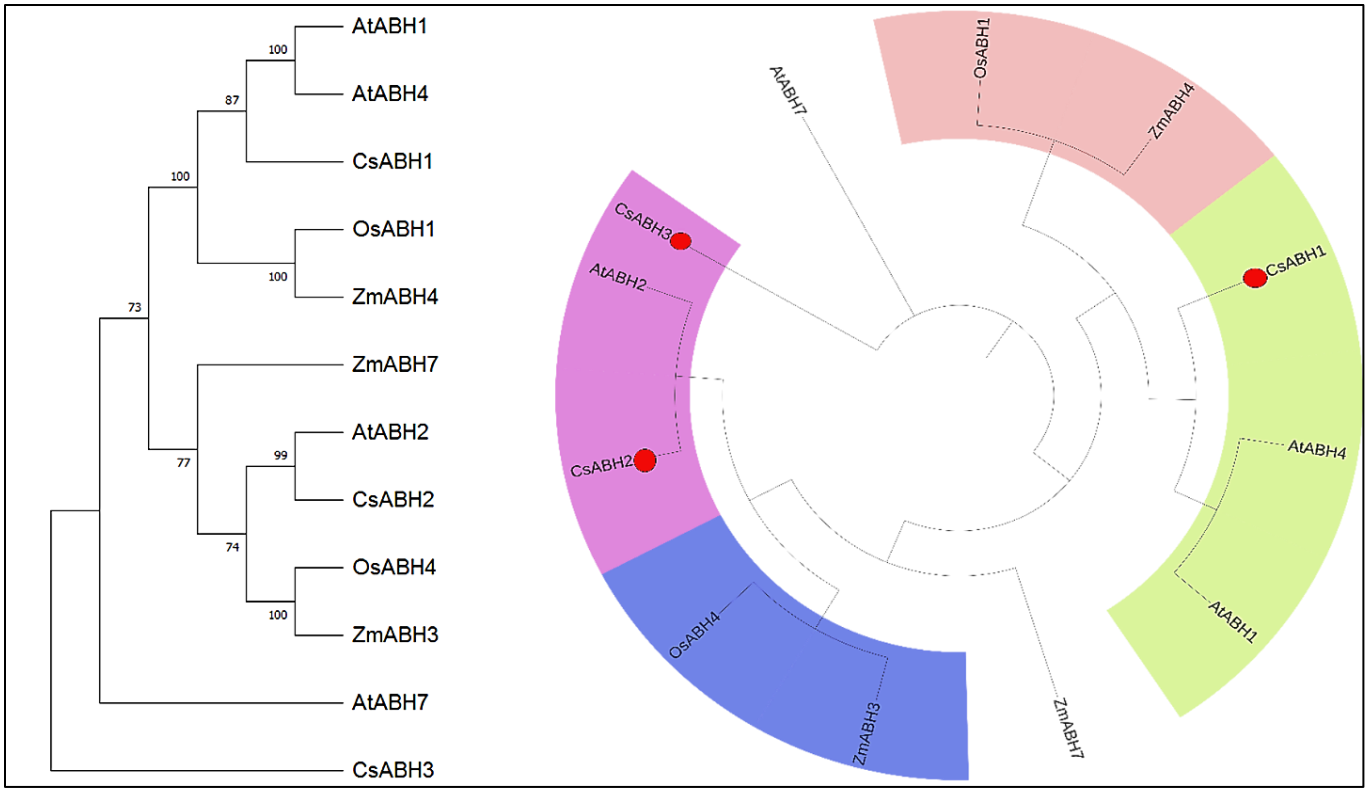


Figure 1. Phylogenetic analysis of Alpha/beta hydrolase superfamilyof oranges with some model plants such as thale cress, rice and corn. Four different clads are shown which could be used to identify evolutionary relationship among diffeerent protiens. *Citrus sinensis* is made distinct by putting red dot behind the name.

Exon intron structure:

The exon intron ratio was identified by using an online server named, GSDS2.0 server (http://gsds.gao-lab.org/). The results of this analysis are shown in the figure 2. The yellow region in the following figure corresponds to exons while black line represents intron. The blue region is 5', 3' flanking region found in the given sequence of EDS1 gene at their respective sites.

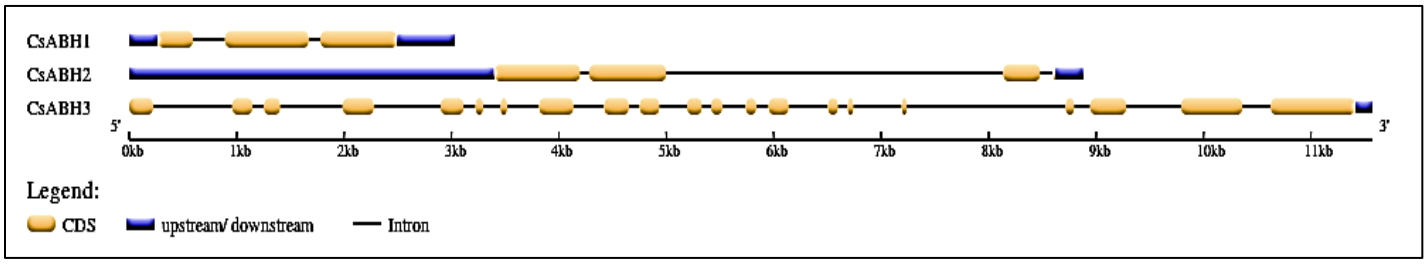


Figure 2. Exon/Intron structure is displayed. Blue is upstream or downstream region while yelllow shows exons and black line is intron.

Conserved domain analysis:

Conserved domain analysis was performed by tBtools on NCBI. The relevant results are shown below. In the following figure it can be inferred that different conserved domains are found in the

given gene including alpha/beta hydrolase super family. Every domain is given a specific color for correspondence. The apha/beta hydrolase super family can also be visualized in dark green color the figure 3.

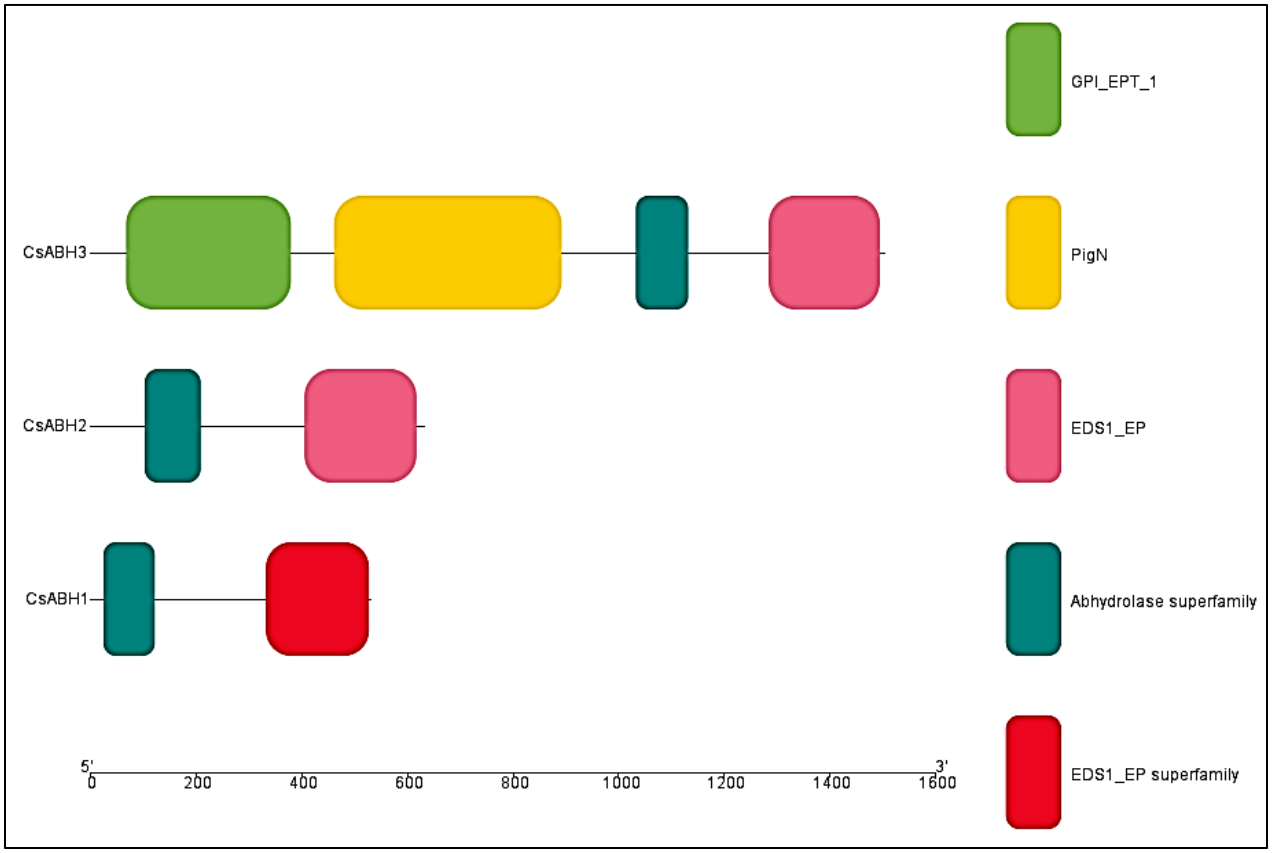


Figure 3. Alpha/beta hydrolase superfamily is found in all three sequences.

Motif analysis:

For motif analysis MEME suit was used with 10 motifs for each sequence and these are shown in figure 4. The motif analysis shows that following consensus sequences are conserved motifs found in all three of peptides under investigation and these motifs are predicted to be a part of Enhanced disease susceptibility 1 gene.

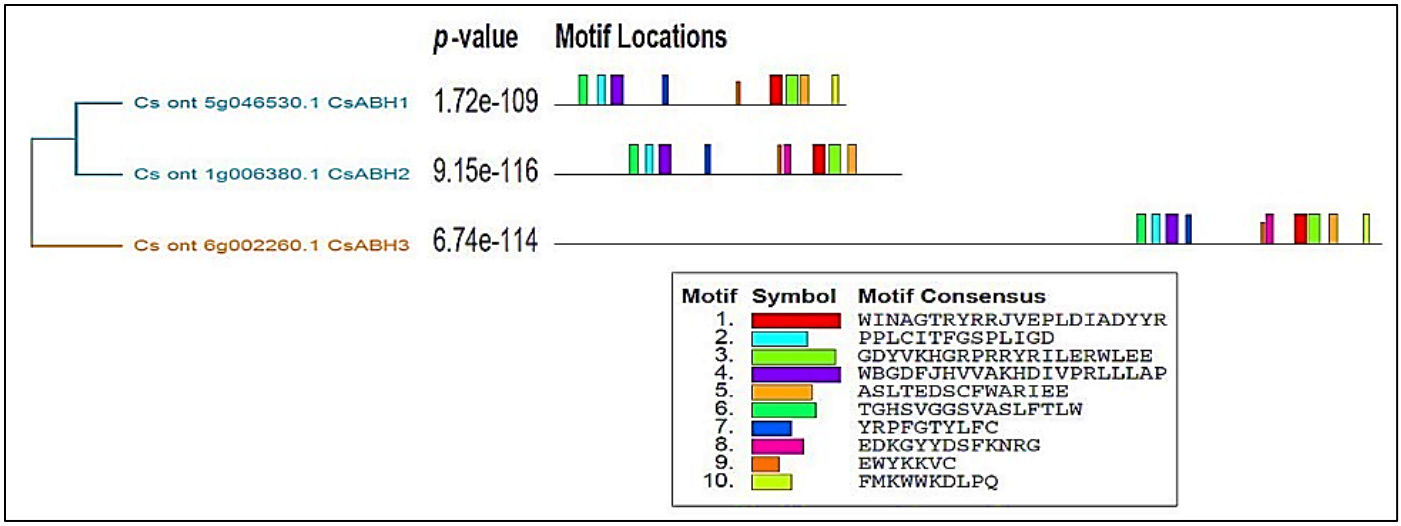


Figure 4. 10 motifs for 3 sequences are shown in this figure. Motif sequences are also given in box.

Sequence analysis of alpha/beta hydrolase:

The respective sequences were analyzed through online ExPasy ProtParam tool. Following details were obtained in this regard including the identification of GRAVY (grand average hydrophobicity index) molecular weight, amino acid number. Furthermore, gene size in base pair, chromosome number, start and end position and other information was retrieved from Citrus Pan-genome to Breeding Database. All of this information is collectively represented in table 1 as following.

Table 1. Physiochemical properties of peptides

Gene Name	Transcript Name	Organism Name	Chromosome	Strand	Gene Start (bp)	Gene End (bp)	Chromosome length	CDS (bp)	Protein length (A.A)	Protein Molecular Weight (kDa)	Pi	GRAVY	No. of EXONS/ INTRONS
CsABH1	Cs_ont_5g046530.1	*Citrus sinensis*	5	+	47,515,965	47,518,997	49,515,363	1,830	530	61.11	7.93	-0.302	3:02
CsABH2	Cs_ont_1g006380.1	*Citrus sinensis*	1	-	1,372,443	1,384,014	25,747,861	1,896	631	71.682	6.54	-0.271	3:02

CsABH3	Cs_ont_6g02260.1	*Citrus sinensis*	6	+	7,089,133	7,098,028	30,300,482	4,518	1505	171.13	7.74	0.038	21:20

Sub-cellular localization:

For sub-cellular localization analysis, the respective amino acid sequences of the proteins translated from the given genes (CsABH1, CsABH2 & CsABH3) were retrieved from NCBI and then subjected to the specified tools to generate the required results. This sub-cellular localization was determined through WoLF PSORT. The descriptive information is available in the table 2. The relevant details were also transformed into heat map through tBtools to better understand the sub-cellular localization of each of the peptide in respective cellular compartments. In this context, the results indicated that all of these proteins were majorly found to be located inside nucleus, while their little presence in cytosol, chloroplast, cytoskeleton, Golgi apparatus and extracellular spaces were also predicted. The relevant table and heat map are shown below.

Table 2. Subcellular localization predicition through Wolf PSORT

Gene name	nucl	chlo	cyto	plas	cysk	golg	extr
CsABH1	5	4	2	1	1	1	
CsABH2	10		3				1
CsABH3	1			13			

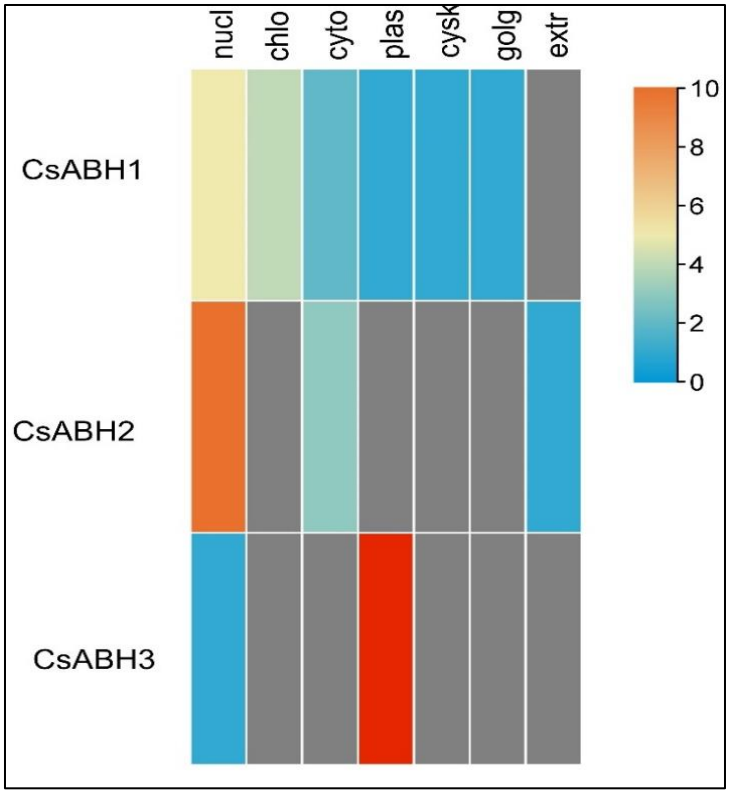


Figure 5. Subcellular localization visualized through heat map. Nucl represents nucleus. Chlo is chloroplast, cyto is cytosol, cysk shows cytoskeleton, golg is golgi apparatus and extr is extracellular. Key is shown along.

Chromosomal location and duplication event:

Three genes were found on three different chromosomes and are displayed with different colours. Synonymous and non-synonymous substitution was calculated through gff3 file via help from tbtools. Moreover, the time of duplication event was estimated through the formula t=ks/2λ ×10-6 where λ=1.5×10-8. In the following table duplication of 2 orthologues from one gene has been depicted. Seq_2 is duplicated from Seq_1. The rate of synonyms substitution per synonymous site is shown by Ks value and similarly number of nonsynonymous substitution divided by nonsynonymous sites is depicted by Ka value in the table value. A statistical test was used to calculate the age of duplication event which employs the use of ratio of Ka to K.

Table 3. Time of duplication event. All three genes are shown in folowing table, 2 genes in Seq_2 benig duplicated from Seq_1 hence all three are mentioned)

Seq_1	Seq_2	Ka	Ks	Ka/Ks	Times (MYA)
Cs_ont_5g046530.1	Cs_ont_1g006380.1	2.836951211	2.785678343	1.018405882	92.85594476
Cs_ont_5g046530.1	Cs_ont_6g002260.1	2.362201033	2.715218198	0.86998571	90.50727327

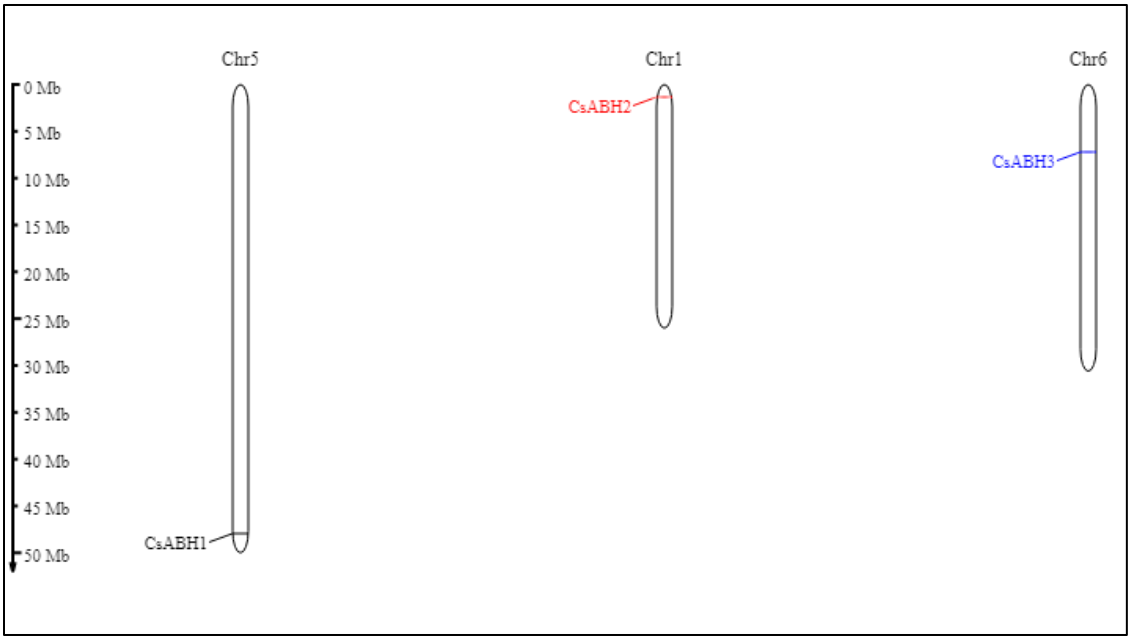
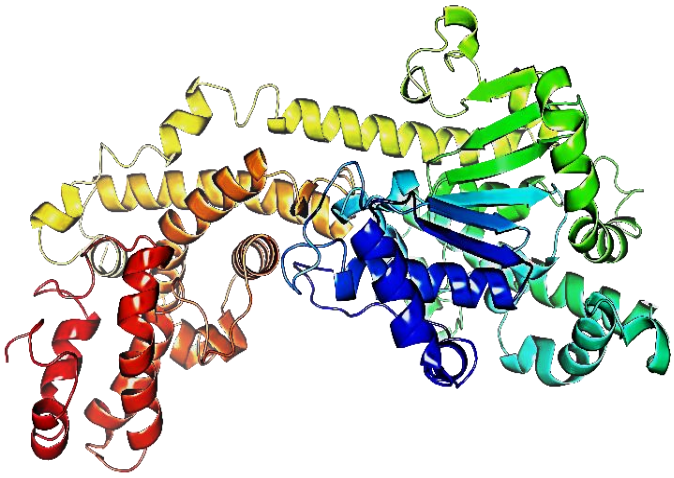
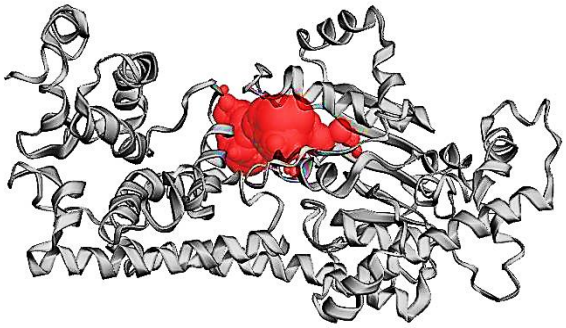


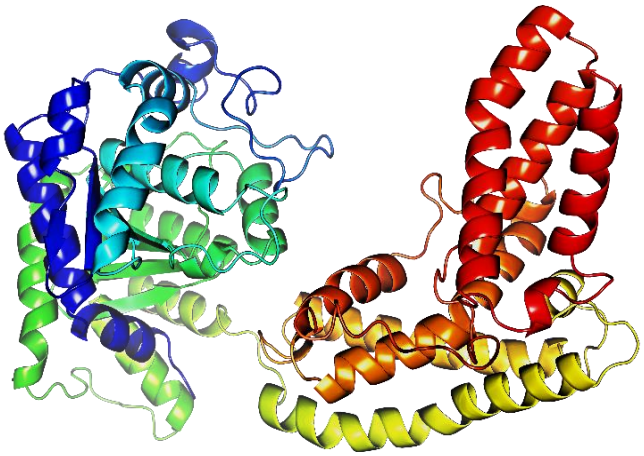
Figure 6. Chromosomal map of Alpha/beta hydrolase superfamily.

Three dimensional (3D) structure and binding sites:

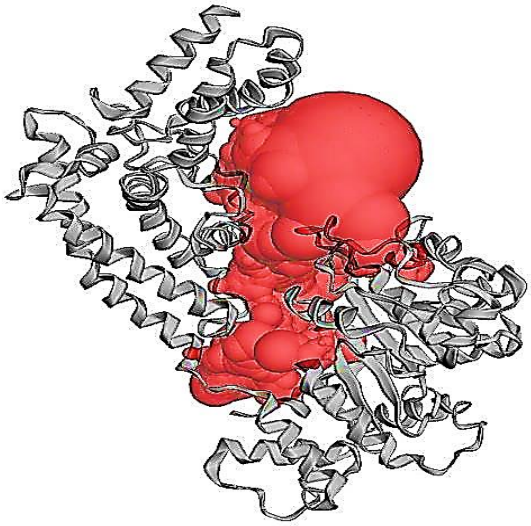
The three dimensional (3D) models of the relevant proteins were predicted by using phyre2.0 web server. The generated results are displayed below in table 4. In this regard, the binding sites of all the 3D models were also predicted to have more insights wherever we tend to discuss their enzymatic activities. The respective pockets in these proteins are presented in red color to make them prominent. These binding sites were predicted with CASTp webserver. This approach will definitely assist in comprehending the structural and interacting characteristic features of these proteins for various enzymatic scenarios. In the following table the respective legends can also be observed in this context, in which the confidence percentages are also shown which affirm the authentication of the constructed 3D models.

Table 4. 3D models and their respective binding sites of CsABH1, CsABH2 & CsABH3 proteins.

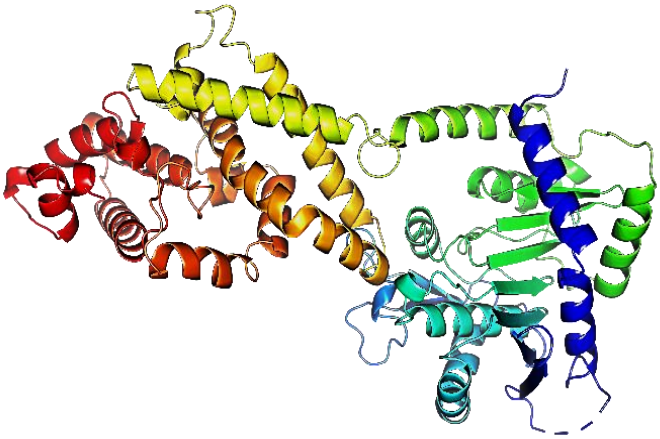
Predicted structures	**Binding sites**
 Predicted structure of **CsABH1**. Coverage was 95% with 100% confidence	 Predicted pockets of **CsABH1**. Radius probe 1.4



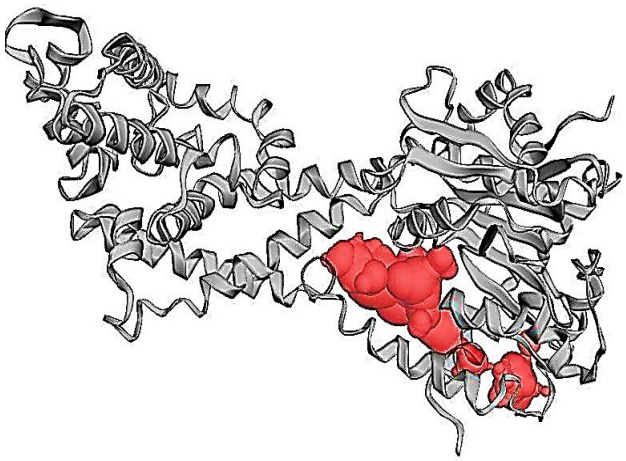
Predicted structure of **CsABH2**. Coverage was 82% with 100% confidence.



Predicted pockets of **CsABH2**. Radius probe 1.4



Predicted structure of **CsABH3**. Coverage was 33% with 100% confidence.



Predicted pockets of **CsABH3**.Radius probe 1.4

DISCUSSION

This study is mainly comprised on the genome wide analysis of EDS1 gene which encodes different peptides mainly, alpha/beta hydrolase. This protein helps in plant defense response in many ways particularly by the synergistic action with PAD4 and NR1 triggering defense response in *Arabidopsis thaliana* [32]. Moreover, certain reported studies have proved the significant

importance of this gene in plant's protection mechanism against certain biotic stress, such as evident by the reported work of Dongus, J. A., & Parker, J. E. (2021) that showed EDS1 carry signals from NLR (nucleotide binding-leucine-rich-repeat) to activate the defense mechanism of plant [33].

On the contrary, some of the variations has also been reported where EDS1 was not as essential as reported in other cases and plant had developed some other pathways and process to confer immunity such as mutated lines of *Nicotiana benthamiana* that do not contain the EDS1 gene still showed pattern recognition behavior [34]. Another study has reported that in duckweed, despite the absence of EDS1 gene, plants showed defensive response in relation to biotic stress but it was mediated by some other mechanisms which do not involve enhanced disease susceptibility gene [35].

In this study we have performed genome wide analysis on EDS1 gene in *Citrus sinensis* which is commonly called as sweet orange. We identified 3 different genes encoding EDS1 in the genome of targeted plant. We preferred the latest genome version 3.0 from CPBD. Performing the phylogenetic analysis, we identified evolutionarily related organisms that showed the similar protein homology. Another genome wide analysis was performed by Aguila et al. [36] on *Citrus sinensis* and the protein they selected was ADF gene encoded, which plays role in plant growth regulation and defensive mechanism. They found 14 different genes in genome of C. sinensis and grouped them into 5 clades. Moreover, expression analysis also showed its importance in plant growth involving symbiotic relation with endophytic organism leading to regulation of this gene in stem, leaves and roots. Similarly, another study was reported by Xi et al. [37] but on WRKY transcription factor. Their results suggested that these transcription factors may be involved in plants protection against infection with *Penicillium digitatum* and other pathogens.

Further, we've also performed the conserved domain analysis suggested the presence of alpha/beta hydrolase in the given sequence of EDS1 gene that play its defensive role in plant protection under stress condition imposed by different pathogens. Chromosomal mapping and location revealed that these genes are located on different chromosome throughout the genome of sweet orange. Moreover, Ka/Ks ratio estimated the age of duplication event. Due to enzymatic nature of protein we also predicted its 3D structure and also predicted its binding sites.

Similar work has also been reported by Liu, K. and Zhou, Y. (2022). They've evaluated the role of trehalose-6-phosphate synthase by performing the genome wide analysis of enzyme and its expression analysis in relation with abiotic stresses and phytohormones [38]. Zhang, Y. *et al.* (2023) scientists has also performed genome wide study on Rboh (respiratory burst oxidase homolog) and evaluated its expression analysis in response to cold stress in sweet orange [39].

In relevance to the current discussion our work shows the importance of EDS1 gene in sweet orange, mediating its role in plant defenses and it may be beneficial for other scientists in better understanding and further evaluating its role. This research may also provide insights into

elaborating and using this gene for other perspectives related to plant defense mechanism. Still further work and analysis are needed to comprehend more about the structural, functional and especially the enzymatic characteristics of EDS1 gene and its respective protein.

CONCLUSIONS

In this study three different genes were identified showing conserved alpha/beta hydrolase super family that play role in plat defense by mediating different pathways such as salicylic and jasmonic acid path ways. Evolutionary relationship shows the homology with Thale cress while gene is also found to be duplicated on chromosome 1, 5 and 6. Moreover, the exon intron structure is also identified. Furthermore, the prediction of 3D structure and its binding sites has also been assessed in order to comprehend its possible enzymatic characteristics. This study would be helpful in evaluating the role of EDS1 in *Citrus sinensis* which could be further explored for the production of better and more disease resistant varieties of sweet orange.

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- **Conflict of interest:** The authors declare that there is no conflict of interest.

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