International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2019; 7(4): 839-844

Received: 22-05-2019
Accepted: 24-06-2019

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Molecular and *in silico* identification of chalcone synthase gene-2 (CHS-2) in sorghum (*Sorghum bicolor* L.) against anthracnose

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Abstract
In India, Sorghum anthracnose is a widespread disease caused by fungi *Colletotrichum* spp. result in severe economic losses. Chalcone Synthase Gene (CHS) expression induced during biotic and abiotic stresses. CHS is a key enzyme of the flavonoid and isoflavonoid biosynthetic pathway. Sorghum synthesizes various flavanoids in response to fungal infection. A ~1300bp fragment Chalcone synthase-2 gene was amplified by PCR from genomic DNA of Sorghum leaf tissue and further sequenced revealed 1213bp sequence submitted to NCBI database name as *Sorghum bicolor* chalcone synthase 2 (CHS2) gene [MK955891.1]. Further CHS-2 protein sequence was also checked for the presence of conserved domain as chalcone synthase superfamily from Pfam database acc. no. Pfam PLN03170 with E-value 0.0. Based on BLASTP result, The CHS-2 proteins was further taken for homology modelling validated with Ramachandran plot showed 94.10% amino acid in most favourable region with 634 amino acid of CHS-2 proteins template using swiss model. These study of CHS gene in sorghum help in understanding the induction and regulation of genes involved in flavonoid biosynthesis for combating against anthracnose diseases in sorghum. This is the first report of isolation of CHS -2 gene in sorghum and homology modelling in south Gujarat region of India.

Keywords: *Sorghum bicolour* L., anthracnose, *Colletotrichum* spp., chalcone synthase and flavonoid

Introduction
Sorghum (*Sorghum bicolor* L.) belonging to the *Poacaea* family and one of the most significant cereal crop in India and worldwide. It mostly utilize for animal feeding, in brewery production, human food and recently time, a future prospective as biofuel resource. In recent times, sorghum is well acclimatized to various abiotic stresses but susceptibility to various biotic stresses mostly anthracnose disease, and result in low productivity. *Colletotrichum* spp., the main causal agent of anthracnose as fungal disease that infects all plant parts and causing significant damages. The severity of anthracnose in sorghum fields largely depend on susceptibility of host and its surrounding environment. (Burrell *et al.,* 2015; Prom, 2017) [3, 15].
Flavonoids and isoflavonoids are important secondary metabolites that aplay vital role in many stages of plant growth and defence mechanism. Sorghum synthesizes the 3-deoxyanthocyanidins, a exclusive class of flavonoid phytoalexins and act as an necessary component of secondary defence mechanisms against pathogen infection. The anthcyanidin aglycones are structurally similar to 3- deoxyanthocyanidins that accumulate in response to light in sorghum. Accumulation of both 3-deoxyanthocyanidins and anthocyanidins is preceded by accumulation of transcripts encoding the well-studied plants-specific type III PKS as chalcone synthase (CHS), a vital enzyme in flavonoid biosynthesis (Dao *et al.,* 2001; Lo *et al.,* 2002) [6, 12]. CHS, or naringenin CHS, is a plant specific polyketide synthase superfamily that catalyzes the condensation of three molecules of malonyl-CoA with one molecule 4-*p*-coumaroyl-CoA yielding naringenin chalcone, which is then rapidly converted into naringenin (flavanone) by chalcone isomerase (CHI) and further synthesized into various flavonoids by the downstream enzymes involved in this pathway. Chalcone synthase (CHS) as secondary metabolites play key roles in the interactions between plants and their surrounding environment. It has been reported that CHS is also involved in pigment formation, insect repellents and plant defences against pathogen attack and exposure to ultra-violet light. Many CHS genes encoding had been characterized from various plant species. The amino acid sequences of chalcone synthase genes present a high degree of sequence resemblance and have been reported in numerous copies in dicotyledonous plants, where up to seven copies have

been identified in several species. In the monocotyledonous grasses, most of the genera studied *i.e. Oryza*, have two copies of the chalcone synthase gene although seven copies have been identified in *Sorghum bicolour* (Lo *et al.*, 1999; Contessotto *et al.*, 2001; Feng *et al.*, 2015; Das and Rawal, 2016; Sanmugavelan *et al.*, 2018; Wang *et al.*, 2018) [11, 5, 8, 7, 16, 18].

In the current study, we uses molecular and computational approaches for *chalcone synthase* (CHS-2) gene characterized from sorghum to understand the function and structure diversity of CHS-2protein in order to exploring metabolite pathway for developing resistant mechanism and minimize the yield losses. This is the first report of isolation of CHS -2 gene in sorghum and homology modelling in south Gujarat region of India.

Materials and methods

Molecular Characterization

Sampling, Identification and extraction of DNA

The primary detection was done by identified the fungus infection in sorghum plants with leaf spot having small red coloured spots on both surfaces of the leaf collected from Main Sorghum Research Station, Navsari Agricultural University, Surat. Total genomic DNA was isolated from sorghum infected leaves using modified CTAB method (Kalaria *et al.*, 2013) [9].

PCR amplification of CHS gene

PCR was performed with some slight modification in parameter for the amplification of CHS gene in sorghum. A pair of chalcone synthase gene specific primers were designed *in silico* using tool FAST PCR (https://primerdigital.com/fastpcr.html) and synthesised as CHSF (CTCGGTGAACCGCCTGAT) and reverse primer as CHSR (GGACATGTTGCCGTACTC AGA) are designed from conserved chalcone synthase gene family. Amplified PCR product was further analysed by 1.5% agarose gel electrophoresis and transilluminator (Lo *et al*., 2012; Das and Rawal, 2016) [7, 12].

Purification and sequencing

Amplified PCR product was further subjected to sanger sequencing with some modification. Retrieved Sequence was further analysed for sequence similarity index using NCBI-BLASTN for the nomenclature of sequence and submitted to NCBI database using BankIt. Phylogenetic trees were constructed using multiple sequence alignment (MSA) in the Clustal_X version 2.0 Software and neighbor-joining method with 1000 bootstrap replications available in the MEGA version 6.0 (Batschauer *et al.* 1991; Kalaria *et al.*, 2013; Conrad *et al.*, 2016) [2, 9, 4].

In silico characterization of CHS protein

CHS protein analysis

The protein sequence was further analyzed for different ORF using ORF finder tool (https:// www.ncbi. nlm.nih.gov/orffinder/) of the NCBI followed by NCBI-BLASTP for Chalcone synthase protein identities. The CHS protein sequence was also check for the presence of conserved domain using NCBI-CDD tool (https://www.ncbi.nlm.nih.gov/ Structure/cdd/cdd.shtml) (Kalaria *et al*., 2013; Feng *et al*., 2015; Conrad *et al.*, 2016; Patel, and Kalaria,2018) [9, 8, 4, 14].

Structure Modelling of CHS Protein

BLASTP based homolog search with RCSB-PDB (PDB; http://www.rcsb.org/ pdb/home/home.do) was also carried out with CHS protein sequence to find out the structural homologs in PDB database. Template sequences with more than 30% sequence identity were downloaded from RCSB-PDB after performing BLASTP. As these procedures yielded one close homologs. So, we choose to go for homology modelling of CHS protein using online swiss model web server (https://swissmodel.expasy.org/) (Awasthi *et al.*, 2016; Conrad *et al.*, 2016; Patel, and Kalaria, 2018; Sanmugavelan *et al.*, 2018) [1, 4, 14, 16].

CHS protein Models Validation

Template sequence in PDB format was retrieved from swiss model web server for visualizing and validation of the model. The stereochemical quality of build model using swiss model was analyze through amino acid region in Ramachandran plot using procheck web server (https://servicesn.mbi.ucla.edu/PROCHECK/) (Awasthi *et al.*, 2016; Patel, and Kalaria, 2018; Sanmugavelan *et al.*, 2018) [1, 14, 16]. Based on the percentage of favourness and frequency of outliers, the models were selected and could be used for further examination.

Results and Discussion

Primary identification of CHS gene in sorghum

During small survey in March, 2019 Sorghum plants with severe anthracnose symptoms were observed in the Main Sorghum Research Station, Navsari Agricultural University, Surat (21°10˙25.1″N and 72°48˙8.1″E). Symptomatic leaves with severe infections of anthracnose in sorghum as reported by many investigators (Burrell *et al.*, 2015; Prom, 2017) [3, 15] were randomly collected and studied further (Fig.1).

Fig 1: Anthracnose symptoms on Sorghum plant exhibiting red coloured spot on leaf, stem of plant (a) and close view of Sorghum leaves (b).

Molecular identification of CHS gene

Randomly five anthracnose infected sorghum leaves were taken for Genomic DNA isolation (Fig. 2).The Genomic DNA was further taken for amplification of CHS gene using specific primer of CHSF and CHSR. The presence of CHS

gene in sorghum DNA can be implied by amplification of ~1300bp fragment (Fig. 3). On sequencing, a 1213 bp long sequence of Chalcone synthase gene was obtained. Feng *et al*. (2015) [8] also isolated the genomic DNA of *Lamiophlomis rotate* and using RT-PCR and RACE-PCR obtained the candidate LrCHS gene sequence of 700bp and 1200bp. Conrad and Mathabatha [4] (2016) amplified CHS gene of 933bp in *Clivia miniata.*

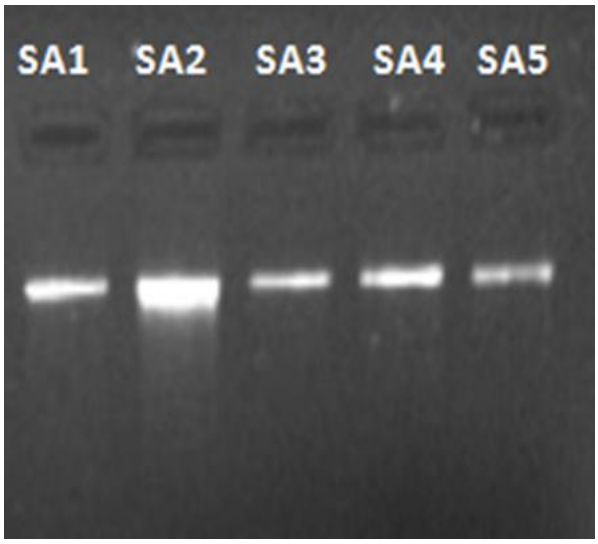

Fig 2: Total Genomic DNA isolation from Anthracnose infected Sorghum

Phylogenetic relationship of CHS gene of Sorghum

BLAST database result showed that all the sequence have more than 99% nucleotide (nt) identities with Chalcone synthase gene reported from worldwide. In BLAST pairwise sequence comparison analysis, obtain sequence has 100 per cent sequence identities with *Sorghum bicolor* chalcone synthase 2 (CHS2) mRNA (MF004347.1), while few matches with other CHS genes type (Table 1). This result clearly indicated that the CHS genes were actively present in sorghum. Accordingly the above result, sequence was named as *Sorghum bicolor* chalcone synthase 2 (CHS2) gene and sequence submitted to Gene Bank (Accession no. MK955891). Multiple sequence alignments of all the sequences were carried out to find the conserved sequences among all. During phylogenetic analysis, CHS gene of sorghum under study (MK955891.1) showed closest relationships with other CHS gene sequences ie MF004347.1, XM 002450830.2, XM 002450826.2, XM 002450828.2, XM 002450827.2, XM 002450832.2, XM 002450825.2, XM 002450831.2, AF152550.1 and AF152549.1 (Fig. 4 A,B). Lo *et al*. (2002) [12] reported different CHS genes in sorghum and sequence deposited in NCBI. Similar work also carried out by Feng *et al.*, (2015) [8] reported CHS gene with higher similarities with *Agastache rugosa* CHS (JQ314450), *Perilla frutescens* (AB002582) and *Perilla frutescens* (AB002815).Conrad and Mathabatha (2016) [4] also performed MSA showed that the *CHS* sequences were highly conserved and shared high sequence identity more than 83% with chalcone synthases from other plants.

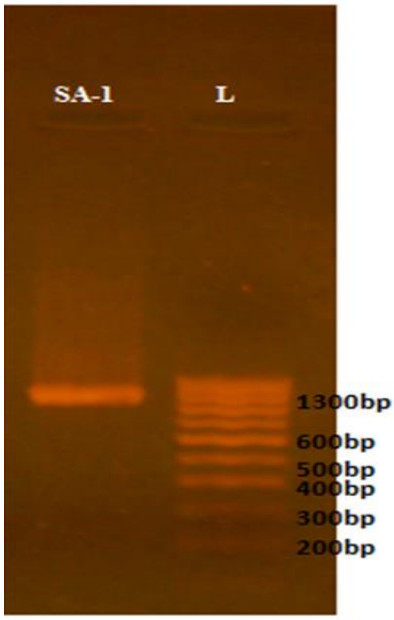

Fig 3: Amplification of CHS gene from Sorghum DNA

Table 1: Percent identities (nucleotide) between Sorghum CHS-2 gene (MK955891) with the other reported worldwide.

Sr. No.	Accession No.	Sequence name	Query Cover (%)	E value	Percent Identity
1	MF004347.1	*Sorghum bicolor* chalcone synthase 2 (CHS2) mRNA	99	0.0	100.00%
2	XM 002449571.2	PREDICTED: *Sorghum bicolor* chalcone synthase 5,mRNA	99	0.0	99.09%
3	XM 002450830.2	PREDICTED: *Sorghum bicolor* chalcone synthase 3,mRNA	99	0.0	96.61%
4	XM 002450826.2	PREDICTED: *Sorghum bicolor* chalcone synthase 2,mRNA	99	0.0	95.37%
5	XM 002450828.2	PREDICTED: *Sorghum bicolor* chalcone synthase 2,mRNA	99	0.0	95.37%
6	XM 002450827.2	PREDICTED: *Sorghum bicolor* chalcone synthase 2,mRNA	99	0.0	95.37%
7	XM 002450832.2	PREDICTED: *Sorghum bicolor* chalcone synthase 6,mRNA	99	0.0	94.71%
8	XM 002450825.2	PREDICTED: *Sorghum bicolor* chalcone synthase 4,mRNA	99	0.0	94.46%
9	XM 002450831.2	PREDICTED: *Sorghum bicolor* chalcone synthase 7,mRNA	99	0.0	94.13%
10	AF152552.1	*Sorghum bicolor* chalcone synthase5 (CHS5) mRNA	99	0.0	99.11%
11	AF152550.1	*Sorghum bicolor* chalcone synthase 3 (CHS3) gene, complete cds	99	0.0	96.27%
12	AF152549.1	*Sorghum bicolor* chalcone synthase 2 (CHS2) gene, complete cds	99	0.0	94.69%

Fig 4(A, B): Phylogenetic analysis of complete CHS-2 gene of sorghum (MK955891) compared with the other reported worldwide.

In silico characterization of CHS-2 protein

In monocots and dicots, the CHS coding sequence are highly conserved. ORF obtain from CHS-2 protein sequence showed the presence of conserved domain as chalcone synthase superfamily from Pfam database acc. no. Pfam PLN03170 with E-value 0.0 (Fig. 5). Contessotto *et al.*, (2001) [5] also found new CHS-2 gene in sugarcane which was further subjected to predicted different ORF to compare with NCBI EST database. Conrad and Mathabatha (2016) [4] also predicted ORF corresponding to the predicted 390 amino acid deduced protein (AEN04070) for CHS genes in *Clivia miniata.* Similar work also had been carried out in Papaya Leaf curl virus coat protein (Patel and Kalaria) [14] and Tomato Leaf curl virus coat protein sequence (Kumar *et al.*, 2012) [10].

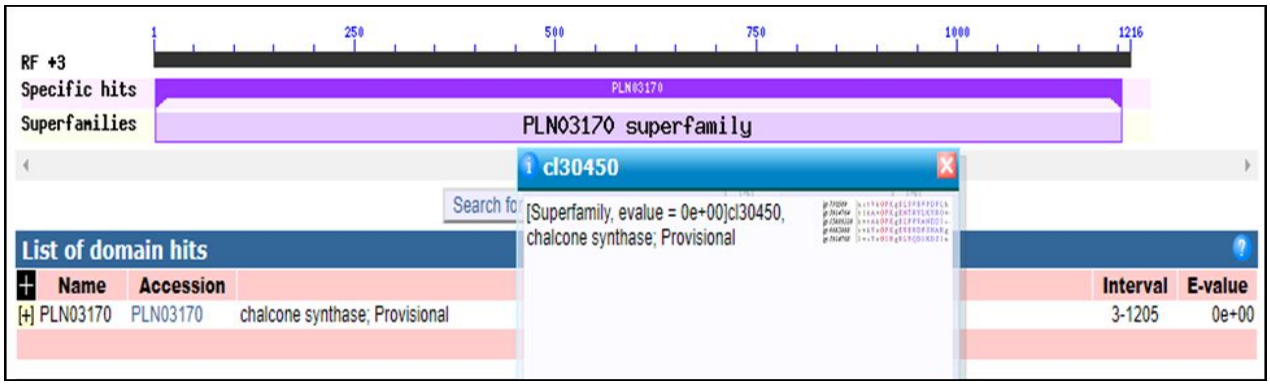

Fig 5: Presence of Conserved domain in CHS-2 gene of Sorghum

Homology Modelling of Coat Protein

BLASTP of CHS-2 protein sequence with PDB database result showed 93.16% similarity with Crystal structure of Type III polyketide synthase from *Oryza sativa* (4YJY.1). Sanmugavelan *et al.*, (2018) [16] also performed MSA of *BrCHS var 2* with others amino acid sequences and further predicted homology template model with reference sequences in PDB as 4YJY.1 by using the YASARA Structure modelling software. CHS-2 protein sequence was further carried forwarded for 3D structure prediction using swiss model web server (Fig 6). QMEAN score obtained 0.51 showed positive sign (Table 2), but the reliability of the structure will depend further on ramachandran plot. Awasthi *et al.* (2016) [1] also performed homology modelling of CHS gene in medicinal plant *Coleus forskohlii* to understand the structure and function of chalcone synthase protein. Similar work also had been carried out by Feng *et al.*, (2015) [8] predicted 3D structure modelled of CHS from L. Rotate. Conrad and Mathabatha (2016) [4] also performed using YASARA software based homology-based modelling of a 3D protein structure of CHS gene in *Clivia miniata.*

Table 2: Homology modelling of CHS-2 protein in sorghum using Swiss Model

Sr. No	Global Quality estimate	CHS-2 protein of Sorghum
1	QMEAN	0.50
2	GMQE	0.98
3	Template	4YJY.1
4	Sequence identity	93.16%

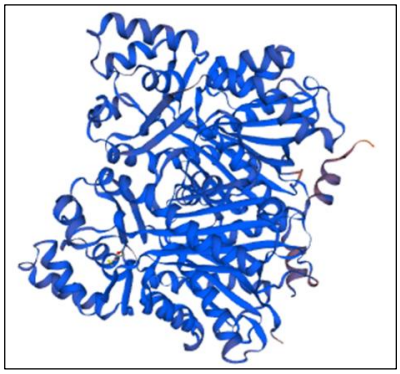

Fig 6: Rasmol visualization of Swiss Model CHS-2 protein structure of sorghum

Validation of Homology Modelling

In homology modelling, assessment of model quality is very critical step. Once the model built, the final model must be examine using validation tools in order to authenticate reliability of model's stereochemistry with typical values originate in crystal structures. Ramachandran plot calculation in PROCHECK tool validation package was used to measured the quality of the modelled structure provided by swiss model calculations (Morris, 1992) [13]. Ideally, one would expect to have over 90% of the residues in these "core" regions to be one of the best guides to stereo-chemical quality (Patel and Kalaria, 2018) [14]. For CHS-2 protein model predicted by swiss model was validated by ramachandran plot. The result revealed 94.10% amino acid in most favarable region with 634 amino acid (Fig 7). Similar work also carried out in for Homology Modelling of Antioxidant Proteins of Spinach (Sahay and Shakya, 2010) [17] and Papaya Leaf curl virus coat protein by Patel and kalaria (2018) [14]. Sanmugavelan *et al.*,(2018) [16] checked the stereochemical quality of *BrCHS var 2* protein predicted model using Ramachandran plot and results indicate that the homology model is of good quality as the number of residues in the favoured region was 92.9%.

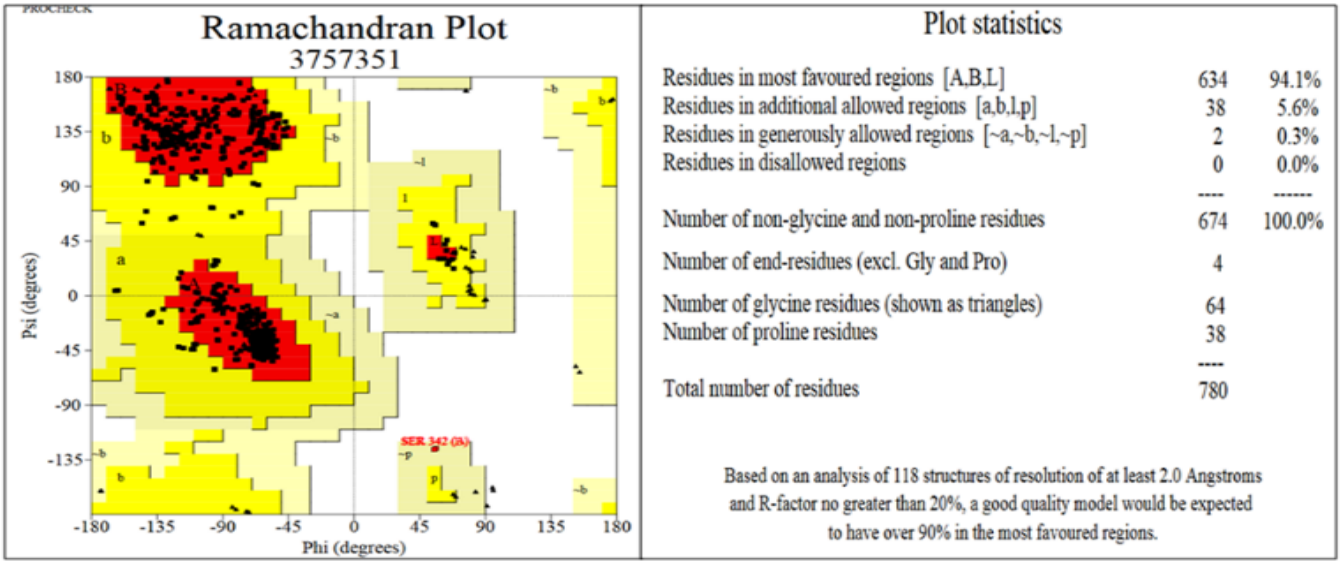

Fig 7: Ramachandran Plot of CHS-2 protein structure of sorghum protein built model using Swiss model

Conclusion

CHS is known as the important enzyme committed to the production of the flavonoid/isoflavonoid biosynthesis pathway has been studied in various species and some of them have several copies of this enzyme (Lo *et al.,* 1999; Contessotto *et al.,* 2001) [5, 11]. It seems that all plants contain at least one CHS gene and often CHS gene families in plant with different expression patterns. Sorghum synthesizes various flavanoids in response to fungal infection. In the present study, molecular identification and *in silico* characterization of CHS-2 gene in sorghum was carried out. CHS-2 gene was amplified from sorghum based on the highly conserved CHS primers sequences and submitted to NCBI database name as *Sorghum bicolor* chalcone synthase 2 (CHS2) gene [MK955891.1] and further CHS-2 protein sequence was also checked for the presence of conserved domain as chalcone synthase superfamily from Pfam database acc. no. Pfam PLN03170 with E-value 0.0. Based on BLASTP result, The CHS-2 proteins was further taken for homology modelling based structure prediction using swiss model and revealed a very good structure with QMEAN score was 0.51 further validated with Ramachandran plot showed 94.10% amino acid in most favourable region with 634 amino acid of CHS-2 proteins template. On based of Ramachandran plot, template have shown near about 90% of residues in core region therefore, predicted model could be place in superior stereochemical quality category. These structures would allow us to evaluate the role of sorghum phytoalexins against anthracnose and for development of new diseases resistance strategy to minimize the yield losses. These result help in understanding the function of the CHS gene and its regulatory mechanism is vital to exploring the genetic control of this metabolite pathway against pathogen. This is the first report of isolation o0f CHS -2 gene in sorghum and homology modelling in south Gujarat region of India.

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