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IN SILICO ANALYSIS, MAPPING OF REGULATORY ELEMENTS AND CORRESPONDING DNA-PROTEIN INTERACTION IN POLYPHENOL OXIDASE GENE PROMOTER FROM DIFFERENT RICE VARIETIES

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Abstract

Polyphenol oxidase (PPO) is an important enzyme that has positive impact regarding plant resistance against different biotic and abiotic stresses. In the present study PPO promoter from six different rice varieties was amplified and then analyzed for *cis*- and *trans*-acting elements. The study revealed a total of 79 different *cis*-acting regulatory elements including 11 elements restricted to only one or other variety. Among six varieties Pakhal-Basmati had highest number (5) of these elements, whereas C-622 and Rachna-Basmati have no such sequences. Rachna-Basmati, IR-36-Basmati and Kashmir-Basmati had 1, 2 and 3 unique elements, respectively. Different elementsrelated to pathogen, salt and water stresses were found, which may be helpful in controlling PPO activity according to changing environment. Moreover, HADDOCK was used to understand molecular mechanism of PPO regulation and it was found that DNA-protein interactions are stabilized by many potential hydrogen bonds. Adenine and arginine were the most reactive residues in DNA and proteins respectively.Structural comparison of different protein-DNA complexes show that even a highly conserved transcriptional factor can adopt different conformations when they contact a different DNA binding sequence, however their stable interactions depend on the number of hydrogen bonds formed and distance.

Key words: Polyphenol oxidase, Promoter, DNA-protein interaction, Regulatory elements.

Introduction

Rice (*Oryza sativa*) produces more food energy per hectare than maize and wheat and is a rich source of protein, vitamins, fiber, minerals, phytochemicals and polyphenols (Pradeep *et al*., 2014). Polyphenol oxidases (PPOs) are special type of enzymes that cause enzymatic browning of different plant parts. These enzymes have a distinct role in the color alteration of steamed bread, pasta and Asian noodles prepared from wheat (Fuerst *et al*., 2006). These reactions have made them an important subject of research in the areas of food sciences as well as plant physiology. The subsequent polymerization of quinones, the products of PPO oxidized phenolic compounds, into melanin pigments results in the enzymatic browning (Nicolas *et al*., 1994). Such enzymatic browning of plant based food products results in the decreased food quality by altering its nutritional properties (Martinez & Whitaker, 1995). Although involvement of PPO in metabolic reactions is not extensively studied, they are applied in resistance against pathogens and insects. Genes for PPOs are present on nuclear genome, whereas its decoded enzyme moves to thylakoids of plastids after being synthesized. The enzyme and its substrates are located spatially separate; therefore reaction proceeds only after tissue damage ruptures subcellular compartments.

Plant PPO gene encodes enzyme that contains two evolutionary conserved domains containing one copper ion each. Therefore these domains are designated as CuA and CuB domains. In each domain a Cu ion has been bound to the histidine molecules. This structure is present in the active site of all PPOs (Marusek *et al*., 2006). Among cereals, PPOs are intensively focused in wheat because they cause drastic discoloration in the products derived from wheat (Massa *et al*., 2007). PPO promoters have been studied in very few plant species so far. Nan-yi *et al*. (2011) studied PPO prompter of Mushroom, *Agaricusbisporus* and found several conserved sequences in it. Additionally they reported TATA boxes, CAAT elements and transcription start site (TSS) in *Ab*PPO promoter. The promoter region also revealed several other conserved elements i.e. AU-rich element (ARE) motif, abscisic acid response element (ABRE), heat shock element (HSE), Methyl and Jasmonic acid (MeJA) responsive motif, and gibberellin-responsive motif. Recently, Shettya *et al*. (2012) amplified and mapped PPO promoter region of eggplant (*Solanum melongena*) and revealed several evolutionary conserved elements. *Sme*PPOPROMOTER contained TSS and consensus Kozak sequence e.g. AGCAATGG. The core promoter boxes i.e. TATA box and CAAT box were present within -90 bp upstream of TSS. Initiator element (Inr) was located +1 bp to TSS, whereas TATA box and CAAT box were mapped at positions -34 bp and -69 bp, respectively. A very less work was made on rice PPO promoters to date, so a study was designed to explore transcriptional regulation of a rice PPO gene.

Materials and Methods

Amplification and sequencing of PPO promoter region: Rice seeds (collected from National Agriculture Research Centre, Islamabad) of six different varieties were grown on wet sterile filter papers at room temperature and DNA was extracted from leaf tissues by following CTAB method (Richards, 1997). A pair of primers was designed using online available program "Primer 3" (http://frodo.wi.mit.edu/), which was used to amplify the upstream region of PPO gene from different

rice varieties. The sequence of the primers is mentioned below;
PPOPF: 5′ CTGGTTCACTTGACAATTTCG 3′
PPOPR: 5′ GCACTGCGCTGTGAACTT 3′
PCR reaction was performed in 25 µl reaction mixture containing 10 x PCR buffer, 25 mM $MgCl_2$, 2 mMdNTPs, 25 pM forward and reverse primer, 30-50 ng / µl template DNA, nanopure water and 1.5 U *Taq* polymerase (Fermentas) using PCR MultiGene Thermal Cycler (Labnet). The conditions followed for PCR amplification were pre-denaturation at 94°C for 5 minutes and 35 cycles each having denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, extension at 72°C for 1 minute and a final extension at 72°C for 20 minutes. The amplified products were sent to Macrogen, South Korea for sequencing after being purified by using Jet quick start purification Kit (GENOMED).

Identification of regulatory elements: Analysis of putative *cis*-acting elements located in the rice PPO gene was initially performed using SIGNALSCAN program in plant *cis*-acting regulatory DNA elements (http://www.dna.affrc.go.jp/PLACElsignalsscan.html).

Molecular modeling: For modeling of *cis*-acting elements, online available bioinformatics tool named "Web 3DNA" (http://w3dna.rutgers.edu/) was used. The structural information for all the putative interacting pairs was gathered using PDB (Berman *et al*., 2000). The template used to perform modeling was selected based on atomic resolution, missing residues and mutation for further protein-DNA docking analysis.

Molecular docking and visualization: Docking was performed using HADDOCK (De-Vries *et al*., 2010). BindN was used for identification of active residues (http://bioinfo.ggc.org/bindn/). Structure files along with active residues of DNA were used for evaluation of models based on HADDOCK Score (De-Vries *et al*., 2010). Graphical analysis of all models was obtained using PyMOL (http://www.pymol.org/).

Results and Discussion

Comparative analysis of regulatory elements: Several regulatory elements found in PPO gene promoter of studied rice varieties were extracted with the help of PLACE database and SIGNALSCAN search tool were used to find the regulatory region. It was observed that the target sequences of all six varieties have 79 different regulatory elements. Among these elements, 11 were unique found only in a single variety (Table 1) and the rest were identified as common that means found in more than one variety. In unique elements, 5 were present in Pakhal-Basmati, 3 in Kashmir-Basmati, 1 in JP-5-Basmati and 2 in IR-36-Basmati. There was no unique *cis*-acting element in C-622-Basmati and Rachna-Basmati. The position of all elements, on plus and minus strands of PPO promoter region in all studied varieties were manually mapped (A representative photograph of all maps; Fig. 1). Some important elements are mentioned below and their proposed functions have already been reported previously. Different studies similar to present investigation have already been reported (Yasmin *et al.,* 2008; Hassan *et al.,* 2015).

Specific elements for anaerobic reactions in animals: Rice PPO promoter region contain two unexpected elements ANAERO1CONSENSUS and ANAERO3CONSENSUS having nucleotide sequence AAACAAA and TCARCAC, respectively. Mohanty *et al*. (2005) reported that these regulatory sequences are involved in the expression of animal genes related to anaerobic reactions.All six studied varieties had 1 copy of AAACAAA, whereas TCARCAC was present only in Pakhal-Basmati having 1 copy.

Table 1. Comparison of unique regulatory elements found in the PPO promoter regions of six rice varieties.

Sr. No.	Element sequence	Element name	Copy Number						Functions
			V1	V2	V3	V4	V5	V6	
1.	ACGTG	ABRELATERD1	0	2	0	0	0	0	Expression of Arabidopsis *erd*1 and rd29a gene in water stress condition (Narusaka e*t al*., 1999; Narusaka e*t al*., 2003; Simpson *et al*., 2003)
2.	ACGT	ACGTATERD1	0	2	0	0	0	0	Expression of *erd*1 gene of Arabidopsis in water stress condition (Simpson *et al*., 2003)
3.	TCARCAC	ANAERO3CONSENSUS	0	1	0	0	0	0	Genes for anaerobic reactions (Mohanty *et al*., 2005)
4.	CACGTG	CACGTGMOTIF	0	2	0	0	0	0	Induction and repression of *phy-A* responsive promoters in Arabidopsis (Hudson & Quail, 2003)
5.	KCACGW	PHERPATEXPA7	0	2	0	0	0	0	Root-hair distribution patterns in rice and Arabidopsis (Kim *et al*., 2006)
6.	WTTSSCSS	E2FCONSENSUS	0	0	1	0	0	0	Cell cycle related genes present in Arabidopsis and rice (Vandepoele *et al*., 2005)
7.	AGGTCA	QELEMENTZMZM13	0	0	1	0	0	0	Expression of *ZM*13 gene of maize specifically in the pollen. It also increased the level of *LAT*52 protein in the pollens of tomato (Hamilton *et al*., 1998)
8.	GAGTGAG	SORLIP5AT	0	0	1	0	0	0	Light induced genes of rice and Arabidopsis (Jiao *et al*., 2005)
9.	MACGYGB	ABRERATCAL	0	0	0	1	0	0	Activation of calcium responsive genes in Arabidopsis (Kaplan *et al*., 2006)
10.	AGCGGG	BS1EGCCR	0	0	0	0	1	0	Expression of Cinnamoyl-CoA reductase gene in vascular bundle of *Eucalyptus gunnii* (Lacombe *et al*., 2000)
11.	CNAACAC	CANBNNAPA	0	0	0	0	1	0	Expression as well as repression of *napA* gene in *Brassica napus* (Ellerstrom *et al*., 1996)

V1, V2, V3, V4, V5 and V6 refer to C-622-Basmati, Pakhal-Basmati, Kashmir-Banaspati, JP-5-Basmati, IR-36-Basmati and Rachna-Basmati, respectively

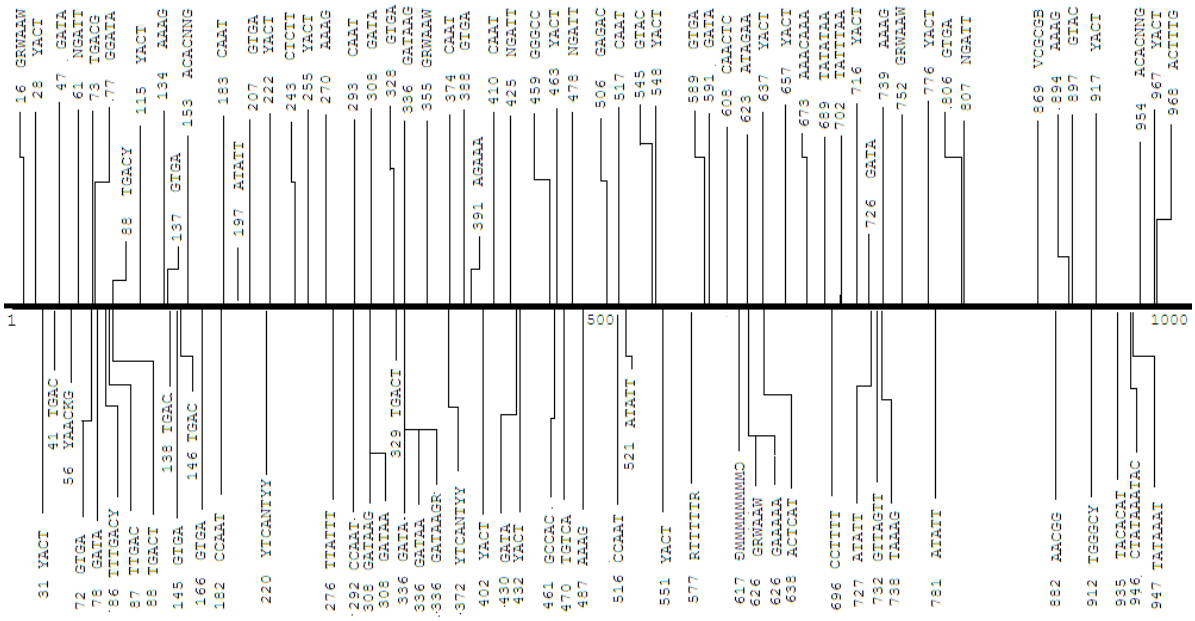

Fig. 1. A representative map of consensus regulatory elements present on the plus strand of C-622-Basmati PPO promoter.

Water stress responsive elements: The studied promoter region contained a hexa-nucleotide element CANNTG. Five varieties including Pakhal-Basmati, Kashmir-Basmati, JP-5-Basmati, IR-36-Basmati and Rachna-Basmati contained this element with copy number 4 or more than it, however this element is absent in C-622-Basmati. According to already reported work, this element is present in the promoter of Arabidopsis *rd*22 gene which responds to conditions related to water stress. It has been reported that this sequence is a binding site for *MYC* transcription factor (Chinnusamy *et al*., 2004). Another motif YAACKG was also found in rice PPO promoter regions with only 1 copy in all six varieties. According to a previous report, Arabidopsis transcription factor *AtMYB*2 (*MYB*-related TF), which is responsive to drought and abscisic acid, can bind to this element and activates the expression of Arabidopsis *rd*22 gene (Abe *et al*., 2008). Beside these elements, rice PPO promoter also contained MYBCORE (CNGTTR). Arabidopsis transcription factors *AtMYB*1 as well as *AtMYB*2 are responsible for the recognition of CNGTTR sequence in water stress related genes (Solano *et al*., 1995). Based on the presence of these sequences in the PPO promoter regions of all studied rice varieties, it can be assumed that PPO gene in rice is responsive to water stress. However such findings can get maturity after performing *In vivo* studies.

Seed specific elements: During analysis of PPO promoter region of different rice varieties it was found that several other important regulatory elements including CNAACAC, CAAT, TATAAAT, TATATAA and TATTAAT are also present. CNAACAC was restricted only to IR-36-Basmati having only 1 copy. Earlier this sequence was found to be involved in controlling the expression of a *Brassica napus* gene *napA* (Ellerstrom *et al*., 1996), which is responsible for encoding a seed storage protein napin. CAAT box in coordination with TATA box and other regulatory elements plays an important role in the expression of pea *legA* gene which is required for encoding a seed storage protein in leguminous plants (Shirsat *et al*., 1989). All six rice varieties were found to have more than 10 copies of CAAT in PPO promoter regions. Different TATA boxes with variations in their sequences such as TATAAAT, TATATAA and TATTAAT were also found. Grace (Grace *et al*., 2004) has reported that TATA boxes are involved in the expression of *Phaseolus vulgaris* (bean) beta-phaseolin gene, responsible for encoding a storage protein in the seeds of bean. It was also revealed that any change in the sequence of TATA boxes can affect the activity these promoter regions. The presence of these elements in rice PPO promoter may have significant role in the expression of PPO gene during the development of rice seed.

Regulatory sequences responsive to hormones: Rice PPO promoter contained another important CAACTC regulatory element which is responsive to gibberellin. Two copies of this element were found in PPO promoter of all rice varieties under investigation. It is a special type of gibberelic acid responsive element GARE known as CAACTC regulatory element (CARE) and is found in the promoter region of rice seed specific gene, cysteine proteinase (*REP*-1). It was also found in the upstream region of barley proteinase gene and rice alpha-amylase gene. It has been reported earlier that *OsGAMyb* transcription factors can bind to these sequences (Sutoh & Yamauchi, 2003).

Presently, TAACAAR sequence in rice PPO promoter region was found once in each studied variety. According to Ogawa (Ogawa *et al*., 2003), it is a gibberellin responsive element during the germination of Arabidopsis seeds. Another important element found in

the present study was CCTTTT, which is present once in each variety. Earlier, CCTTTT (pyrimidine box) region has been reported in the promoter region of barley hydrolase gene. In response to gibberellin, *BPBF* transcription factor recognizes this element and causes the expression of hydrolase gene after seed germination. This pyrimidine box, reported by Morita *et al*. (1998) is found in rice alpha-amylase (*RAmy*1A) gene as well as barley alpha-amylase (*Amy*2/32b) gene. In rice, this box in combination with GAREis involved in sugar repression where as in barley *Amy*2/32b gene it shows positive response to gibberellin.

A penta-nucleotide element TGACG and a hexa-nucleotide element VCGCGB were also found in rice PPO promoter regions. There was only 1 copy of TGACG in every variety whereas copy number of VCGCGB was 2 each for C-622-Basmati, Pakhal-Basmati and Kashmir-Basmati while 4 copies were identified in JP-5-Basmati, IR-36-Basmati and Rachna-Basmati. TGACG has been found in the upstream region of histone (*H*3) gene of wheat and transcription factors (*HBP*-1) can recognize this element. Some transcription factors of *TGA* family of tobacco can also recognize this element. It has been reported that this element is involved in the regulation of many plant genes in response to auxin and salicylic acid applications (Despres *et al*., 2003).Another element VCGCGB was first reported by Yang & Poovaih (2002) and it was found in the promoter of genes related to the light, ABA and ethylene signaling. These are found to be present in multiple copies and recognized by *Arabidopsis thaliana* signal responsive (*AtSR*) factors to activate the expression of the target genes. The fact that calmodulin-binding proteins are involved in the recognition of CGCG boxes provided the clue about the possibility of these proteins being encoded by *AtSR* genes.

Pathogen associated elements: The analysis of rice PPO promoters revealed the presence of a*cis*-acting element having TGAC sequence. Previous studies have shown that TGAC is a target sequence for the binding of *WRKY*71 factor which represses the pathway of gibberellin signaling. This sequence is also reported in the promoter region of *Amy*32b gene. Eulgem *et al*. (1999) worked on Pathogenesis-Related *PR*-10 (Class10) gene promoter in Parsley and found that *WRKY* proteins recognize W-boxes containing TGAC sequences. YTGTCWC is another target site of transcription factors and single copy of this element was found in the PPO promoter region of studied rice varieties except Rachna-Basmati. In a report, Boyle & Brisson (2001) proved through their experimental work that the promoter region of*PR*-10a gene of potato tuber contains this binding element recognized by silencing-element-binding-factor (*SEBF*). This factor, after binding to the target element represses the expression of *PR*-10a gene involved in plant resistance against pathogens. PLACE database also revealed the presence of GAAAAA in rice PPO promoter region of selected varieties for the present study with same copy number i.e. 2 in all varieties. Moreover it has already been revealed that Soybean *SCaM*-4 (*CaM* isoform) gene promoter contains this *GT*-1 motif recognized by *AtGT*-3b (an Arabidopsis *GT*-1-like factor) and these elements are reported to be responsive to salt and pathogen stresses (Park *et al*., 2004). On the basis of above mentioned findings and reports it can be claimed that rice PPO gene expression level may also be affected in response to pathogen attack.

Transcription activation associated elements: Present study revealed another transcription factor binding site having nucleotide sequence GGATA. All varieties had one copy each except Pakhal-Basmati that lacked this element. It is a core element for the binding of *Mybst*1, a homolog of *MYB* proteins. *Mybst*1, in contrast to other plant *MYB*s, contains a single DNA binding repeat instead of two. This transcription factor is involved in transcriptional activation (Baranowskij *et al*., 1998). Moreover, two of the rice varieties (Pakhal-Basmati and Kashmir-Basmati) also contained TGCAAAGin the PPO promoter region. It is known as prolamine box and earlier it was identified in the promoter region of *Glu-B*1 gene of rice responsible for quantitative regulation of the target gene (Wu *et al.,* 2000). These facts support the idea that these elements may have important roles in the regulation of rice PPO gene.

Phytochromeresponsive sequences: Previously, Hudson & Quail (2003) has provided evidence that CACGT in combination with other elements plays a significant role in the induction as well as repression of *phy-A* responsive promoters in Arabidopsis. It is called as G-box and act as a binding site for G-box binding factors (*GBF*s). This element was identified in the studied PPO promoter of Pakhal-Basmati only. Moreover, another evolutionary conserved CGGATA sequence was also identified in the PPO promoter region of studied varieties. Degenhardt & Tobin (1996) have reported CGGATA (*REbeta*) in the promoter region of one of the light harvesting chlorophyll-b (*Lhcb*) genes of *Lemnagibba*. This element is responsive to phytochrome. GATA sequence can also work as an alternative for *REbeta* element. PPO promoter from studied rice varieties except Pakhal-Basmati contained one copy each of such element.

Interactions of TFS with regulatory elements: To understand the regulation of gene expression, studying interactions between protein-DNA will be of great value (Si *et al*., 2011). In the current study, nine TFs were selected to dock for identification of protein-DNA interactions. As prior reports of protein-DNA interactions profiles indicated that maximum residues of DNA and protein interactions are hydrogen bonds (Angarica *et al*., 2008), therefore present study was mainly focused on hydrogen bonding residues among them. The results generated by HADDOCK are summarized in Table 2.

Analysis of molecular docking finding: In the present study, computational methods involving structural analysis of different TFs and DNA modeling have been utilized to understand the transcriptional regulation of PPO gene. In addition, different conserved protein residues along with their interactions with DNA were also evaluated.

Table 2. HADDOCK web server results. Rows are representing the information about models of protein-DNA complexes and their corresponding energies. For each TF, binding affinities with three DNA binding sites are shown. HADDOCk score is combination of buried surface area, Van der walls, electrostatic, de-solvation and restraint violation energies.

Protein / DNA	HADDOCK Score	Cluster size	RMSD (A*)	Vander waals energy (Kcal / Mol)	Electrostatic energy (Kcal / Mol)	Desolvation energy (Kcal / Mol)	Restrains violation energy (Kcal / Mol)	Buried surface area
TEF-1 _ CACATTCTTCAT	-38.4	22	8.9	-67.4	-773.3	24.1	1596.1	1912.4
SOX17 _ GATATTGTT	-154.5	83	1.5	-85.2	-526.7	9.2	268.1	2229.9
c-REL _ TGTGTTTTCC	-68.4	4	1.5	-69.8	-509.1	42.1	611.8	2260.3
Pbx _ CTTGATTGATGT	-96.2	8	2.5	-71.9	-498.3	22.5	528.1	1993.6
SP1 _ GTGGTAGTGT	-100.0	22	7.6	-50.1	-283.9	5.8	11.4	981.5
GATA-1 _ GGATGG	-93.6	6	1.7	-59.5	-210.9	4.9	30.6	1497.5
TBP _ CTATTAATAGAAAAA	-109.0	45	0.9	-47.8	-636.4	25.5	405.5	1625.3
E74A _ TTTCCTC	30.8	39	5.8	-44.2	-355.7	14.2	1320.5	1180.0
HFH-1 _ TAATGTATAGT	-141.0	9	1.5	-73.6	-480.8	11.5	172.8	1892.7

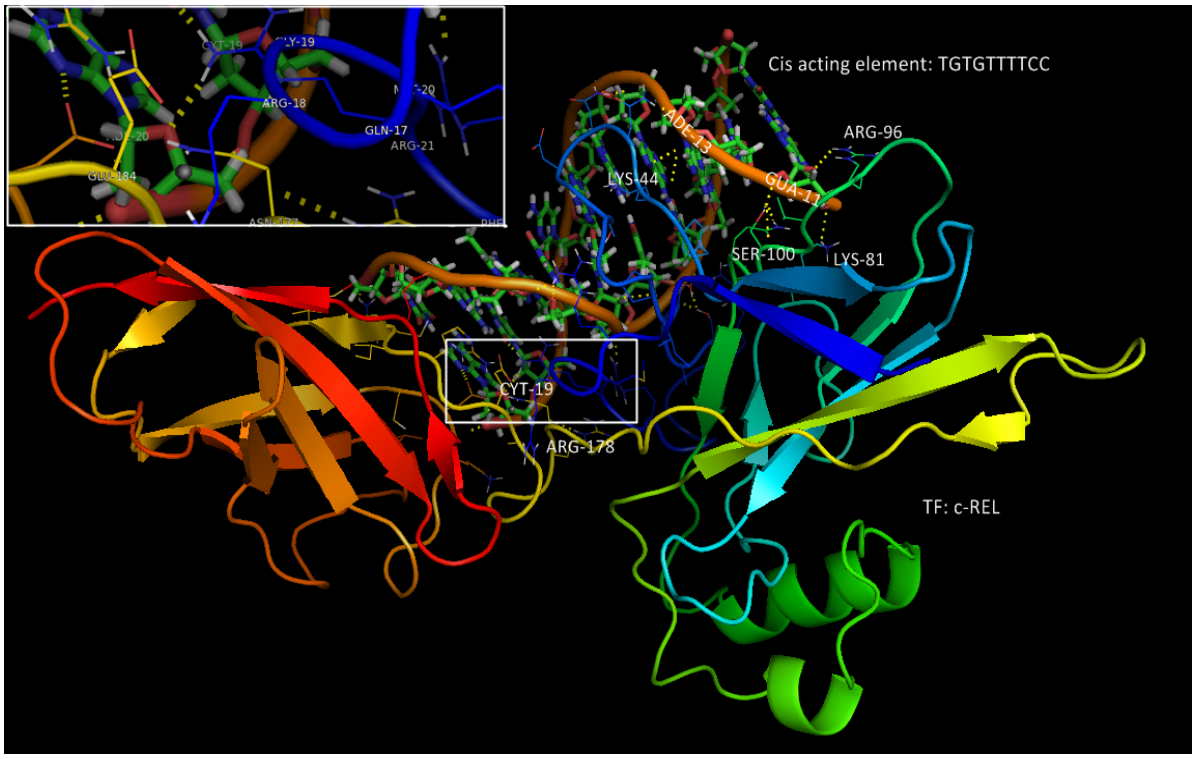

Fig. 2. A representative model for interaction of TF 'c-REL' with regulatory element having DNA sequence TGTGTTTTCC.

The transcription factor c-REL interact with regulatory element 5'-TGTGTTTTCC-3' by making hydrogen bonds between them as shown by yellow dots in the representative structural models of all complexes (Fig. 2). Minimum number of dots between residues represents smaller distances showing strong bonds between them and *vice versa*. GUA11 has made hydrogen bond each with LYS81, ARG96 and SER100 (GUA11…LYS81, GUA11…ARG96 and GUA11…SER100). ADE13 forms two hydrogen bonds with LYS44. Furthermore, ARG21 was also found constructing two hydrogen bonds with CYT19 and ADE20 respectively. In addition, CYT19 is also linked by formation of another hydrogen bond with ARG178. The studied c-Rel monomer binds 5'-TGTGTTTTCC-3' in a similar way as already observed in the case of c-Relhomodimer crystallographic complex identified by Huang *et al*. (2001). Structural information about all other protein-DNA complexes was also analyzed in term of hydrogen bond formation.

Binding studies revealed that the DNA binding domain of a conserved winged helix protein HFH-1 recognizes the TAATGTATAGT with higher affinity as compared to other reported TFs which is indication of stable interaction between them. Particular recognition of the TAATGTATAGT by HFH-1 results from the direct contacts between DNA and protein as well as intrasubunit interactions between winged helix binding motif and the DNA-contacting helix 3 (H3) of the C-terminal domain. A large number of DNA binding proteins have winged helix motif initially identified in the gene products of the Drosophila hepatocyte nuclear factor 3 (HNF-3) and homeotic forkhead (fkh1) that functions as the DNA binding domain of many TFs. The winged helix DNA binding motif contains two flexible loops termed wings, a three-stranded β-sheet, and three or four ∞-helices (Sheng *et al*., 2002). DNA major groove is primarily recognized by Helix 3 and DNA contacts is further stabilized by the two wings. Adenine was found the most sensitive residues

in regulatory elements while in TFs the most reactive residues were arginine followed by lysine. Protein-DNA residues having distance less than 3.5 Å were assumed to be realistic for hydrogen bonding (Coulocheri *et al*., 2007). Therefore, evaluation of hydrogen bond formation is essential because these hydrogen bonds contribute significantly to the stability and specificity of a protein structure (Coulocheri *et al*., 2007).

Differences of regulation in TFs can be predictable by its binding mechanism to DNA (Sheng *et al*., 2002). Our results show that the evident variations occur in studied TFs indicating few *cis*-acting elements, which can form relatively more stable bonds, will primarily be involved in PPO gene regulation. The structural comparison of DNA complexes and different TFs indicates that even highly conserved TF can adopt other local structures when they bound to another DNA binding sequence, while their stability and interaction depends on the number of hydrogen bonds formed and distance.

Conclusion

PPO is an economically important enzyme that proves drastic effects regarding plant products. On the other hand, it also plays significant role in plant resistance against pathogens and other biotic and abiotic stresses. The study revealed different *cis*-acting elements which may be responsible for specific functions. Among these elements, there were two unexpected sequences i.e. AAACAAA and TCARCAC that are specific for anaerobic reactions in animal cells. The appearance of unique as well as common elements proves that PPO promoter region can affectively be utilized for a wide range of activities like biotic and abiotic stresses, hormone induction and pathogen resistance etc. It can also be concluded from present study that the interaction among regulatory DNA regions and their corresponding TFs are stabilized by many potential hydrogen bonds.

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