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Molecular cloning and characterization of cm3 gene, from t. Aestivum and t. Durum genome

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***Abstract*-** Alpha-amylase inhibitors from wheat are found noble sources to develop insect resistant transgenic plants. Thus far wheat alpha – amylase inhibitor genes have been cloned, sequenced and characterized by various researchers through c-DNA library. A subunit of tetrameric alpha amylase CM3 gene was isolated and characterized directly from genomes of different Indian wheat varieties using PCR approach. We have screened different Indian wheat varieties for alpha amylase inhibitor protein(s) and isolated the gene from selected variety to develop transgenic plant having gene of better expressivity and enhanced activity. The alpha amylase inhibitor gene encoding CM3 protein, from *T. aestivum* and *T. durum* has cloned and sequenced, the gene sequences from the both species were found same except at one position which is out of coding region. Further, characterization reveals that the isolated gene is intronless and conserved throughout the different varities of *T. aestivum* and *T. durum*. It may be possible that there is no ecological or geographical effect on the gene. This indicates probability of important biological role for this protein in wheat.

Index Terms- Alpha amylase inhibitors, touchdown-PCR, alpha amylase/ trypsin bifunctional inhibitor (CM proteins) genes.

I. INTRODUCTION

Wheat-α-amylase inhibitor was first reported in the endosperm by **Kneen and Sandstedt (**1**),** latter it was reported in the gliadin (**2)** and albumin fraction **(3)** of wheat kernel.

Wheat alpha amylase inhibitors are tetrameric, dimeric, and monomeric in nature and, can be fractionated by means of gel filtration into three isoinhibitor families with apparent Mr. close to 60,000, 24,000 and 12,000 (**4**) respectively. According to their relative gel electrophoretic mobility they also called inhibitor 0.45, inhibitor 0.19 and inhibitor 0.28 (**5, 6**). The presence of multiple amylase inhibitors with Mr close to 60,000 was first reported by Petrucci, T. in 1974(7). These multiple amylase inhibitors are tetrameric and soluble in chloroform: methanol mixture and termed as CM proteins (**8**). Wheat CM proteins belong to the trypsin/ alpha-amylase bifunctional family. These proteins are encoded by multigene family and dispersed over different chromosomes **(10)**. The tetrameric inhibitor is composed of three types of subunits CM2/CM1; CM16/CM17 and CM3 proteins and all are required to obtain fully active inhibitor protein **(9)**.

Different wheat alpha-amylase inhibitors genes have been isolated and characterized using cDNA library approach (**11, 12, 13, and 14**). In the present study an attempt is made to isolate alpha-amylase inhibitor gene(s) from different Indian wheat varieties, to study the deference in inhibitory activity within or between the species, and their gene pattern, as well as polymorphism

II. EXPERIMENTAL

1. Plant material

The study was performed on 10 Indian wheat varieties *viz*. Kundan, Sonalika, UP1109, UP2003, UP2382, UP2425, UP262 genotype from species *T. aestivum*, PDW 276, PDW274, PDW279 genotype from species *T. durum.*

2. PCR amplification

Isolation of genomic DNA was done by Delaporta *et al. method (*1983). PCR amplification was performed in 50µl volume which was consisted of 100 ng of genomic DNA, 3 units taq DNA polymerase, 200µM dNTPs, 1X PCR buffer and 1.5 mM $MgCl_2$. In the present exercise a modified form of 'Touchdown PCR' (15**)** was done to resolve the amplicon(s) of desired length.

The cycling parameters were 95^0 C for 5 min to pre-denature followed by 30 cycles of 95^0 C for 1 min, 50^0 C for 2 min, 72^0 C for 2 min, and a final extension at 72^0 C for 10 min., amplification product were separated on 2% agarose.
Oligonucleotide primers were custom-synthesized by Genei India.

Forward-$_{5'}$ d ATGGCGTGCAAGTCC $_{3'}$
Reverse-$_{5'}$ d GCATACATGCACACCAC $_{3'}$

3. Cloning and screening of PCR fragment

The desired DNA fragments were gel purified and ligated to pGEM-T easy vector (Promega) and transformed in E.coli DH5α. The positive clones from different varieties were screened by restriction digestion and dot blot. The selected clones were sequenced, using ABI PRISM automated sequencer.

4. Southern Blotting

Genomic DNA from *T. aestivum* and *T. durum* was digested with EcoRI and subjected to Southern blotting by using pTd13.39 as a probe. Southern Blotting was done onto positively charged nylon membrane with high salt (**16**). Detection of southern blot was done using Ambions Brightstar^{TM} detection kit, with slight modification.

III. RESULTS AND DISCUSSION

Thus far different alpha-amylase inhibitor genes have been isolated from cDNAs library (**17, 18**) and well characterized. In present work CM3 genes from different varieties were isolated directly from genomic DNA using touchdown PCR approach.

1. Isolation and cloning of gene

Genomic DNA from different varieties of *T. aestivum* and *T. durum* were isolated and subjected to Touchdown PCR amplification. A single band of ~580 bp was resolved at annealing temperature of 50^0C in all wheat varieties (fig.1).

The amplicons of ~580 bp were purified by gel extraction and ligated to pGEMT easy vector (3.015 kb) followed by transformation in DH5α. The transformed colonies were screened on the basis of blue white selection. The plasmid from different positive clones were isolated and screened by EcoRI digestion, fragments of ~580 bp and 3.015 kb were recovered after digestion as the gene of interest was flanked by EcoRI site in the cloned vector. The purified amplicons were subjected to dot blot analysis using pTd 13.39 probe, and all varieties were found positive for CM3 specific gene. The positive clones from varieties UP2425 and PDW279 showed highest color intensity (fig.2).

2. Sequencing and characterization of the gene

The positive clones from varieties UP2425 and PDW279 corresponding to *T. aestivum* and *T. durum* respectively were selected on the basis of dot blot analysis and sequenced using AB1 PRISM automated sequencer.

The size of gene insert was found 575 bp as shown in Fig. 4. The two clones were named as pTa2425 and pTd 279 (Gene accession No. AY436554, and AY465898 respectively). The gene sequences of both the clones were identical except at one change at 545 nucleotide residue which was found beyond the coding region. The two gene sequences showed identity with the mRNA of CM3 protein from *T. aestivum* and *T. durum* a subunit of tetrameric alpha-amylase inhibitor protein of wheat **(17, 18)**.

3. *Sequence analysis of gene clones pta2425 and ptd279*

The 575 bp gene include an open reading frame of 504 nt from 1 to 504 bp and flanked by 74 nt 3′ non-coding tail. The open reading frame encodes protein of 168 residues in which N terminal 25 amino acids include signal peptide. The mature protein consists of 143 amino acids and molecular mass of 15.8 kDa. Coding region had 59.7% G+C content (Fig. 4).

4. *Comparison of gene sequences*

The comparative study of gene sequence was done using BLASTN 2.2.6 programme present on the NCBI website. The gene sequences showed 100% homology with wheat m-RNA coding for CM3 protein (**17,18)** , 90% with mRNA encoding CMd of barley (**19**), 89% homology with HVCMDAA1 gene encoding CMd, 91% of CMd gene, 89% with CMd3 gene, of barley (**18**), 83% with wheat CM16 mRNA (**17**, **20**), 70% with wheat CM17 mRNA, 87% with mRNA encoding CMa of barley (**19)**, 82% with BTA12 mRNA coding for CMb of barley **(19)**, 72% with CM1 (**17**) encoding mRNA and 66% with CM2 mRNA (Table 1). It also showed 85% and 81% homology with *Elucine coracana* alpha amylase- trypsin bifunctional inhibitor gene and Hageman factor inhibitor gene respectively.

5. Comparison of CM3 protein with different alpha-amylase inhibitors and trypsin inhibitors

The protein homology search for deduced CM3 protein was done using BLAST protein –protein alignment programme present on NCBI website-BLASTP 2.2.25

The homology study has revealed that there are 42-88% amino acid sequence homology present between CM3 protein to other CM proteins from wheat and barley. CM3 protein shows 83% homology with the CMd of barley (*H. vulgare),* 51% homology with CM16 protein of wheat, 50% with CMb protein, 45% homology with CMa protein, 38% with CMc protein, 38% with CMe of *H. vulgare*, 49% with CM2 of wheat, and 42% homology with CM1 protein. It shows 43% homology with Hageman factor inhibitor of maize, (Table 1). Maximum homology between CM3 and CMd reveals that might be they have identical function in their respective tetrameric protein.

It is evident that CM3 primary structure is very close to trypsin or bifunctional alpha amylase inhibitors from different sources than to alpha-amylase inhibitor of wheat itself. It shows 37% homology with alpha-amylase /trypsin inhibitor of *Elucine coracana* (RBI) and 34% homology with alpha-amylase/ trypsin inhibitor of barley, while only 28% homology with dimeric 0.53 and 0.19 AAI of wheat and only 25% homology with wheat bifunctional trypsin/ alpha-amylase inhibitors CMX2 and CMX1/CMX3 (Table 1).

6. Amino acid composition and characterization

Leucine is most abundant amino acid in CM3 followed by Ser and Pro the data comes from the primary structure deduced from gene sequence as shown in Fig 4.

The mature protein sequence of CM3 contains twelve cysteine residues, out of these 9 are conserved which is a characteristic feature of CM proteins. These conserved residues include a double Cys-Cys residue followed by a consensus sequence of Cys-Arg-Cys (CRC), nine residues apart from it. These 9 Cys residues are involved in disulfide bonds and are organized in a conserved cysteine motif that may have an important functional and structural role for the CM proteins.
Comparative analysis of different wheat tetrameric alpha amylase subunits reveals that the N-terminal signal peptide sequences are highly conserved among them. The N-terminal residues from 40-50 amino acid of mature protein show great homology, while CC and CRC residues are conserved (Table 2). These conserved sequences may accounts for stable quaternary structure and activity of the tetrameric protein.

Further homology search reveals that CC (Cys-Cys) doublet was found in all storage proteins including RBI,0.19 alpha amylase inhibitors and 0.53 AAI, while CRC sequence is conserved in RBI and 0.19, alpha amylase inhibitor. The most conserved sequence found in CM proteins is DLPGCPRE that is situated between 126 amino-acid to 133 amino acid residue of CM3 protein. It is also conserved in RBI and Hageman factor inhibitor. This conserved sequence is not found in wheat 0.19 AAI and 0.53 AAI and may be the characteristic sequence of alpha amylase/ trypsin - bifunctional inhibitor.

7. Secondary structure of CM3 protein

The secondary structure of CM3 protein were analyzed and their consensus secondary structure was predicted by different methods (DPM, DSC, GOR4, HNNC, PHD, Predator, SIMPA96, SOPM) main with the analysis programme NPS. The CM3 protein has four main secondary structural units: α-helix, random coil, extended strand and ambiguous state while random coils occurred most frequently in the above structures.

8. Southern analysis of cm3 gene in T. aestivum (cv. UP2425) and T. durum (cv.PDW 279)

Southern blotting of wheat genomic DNA was used to investigate the organization and copy number of gene(s) encoding the *T. durum* CM3 protein and *T. aestivum* CM3 protein. High molecular weight DNA from cv. PDW279 and UP2425 was digested to completion with EcoR1 (Fig. 3). Single fragment of approximately 23 and 24 kb respectively was detected in both varieties with probes corresponding to CM3 proteins. Since there is no EcoR1 site present in the CM3 encoding gene, the number of restriction fragments gives a minimal estimate for the gene copy number present in the both varieties. The sequence of CM3 encoding gene reveals there is only one ORF encoding the entire protein. There is no evidence for the presence of introns in the sequence, which is consistent with the analysis of barley CMd genes (**18**).

Sequence comparison reveal that the gene sequence of CM3 protein, of the two Indian cultivars from *aestivum* and *durum* species are similar to the gene sequences of Chinese spring and agathe of *T. aestivum* and *T. durum* respectively (**21**). This further confirms that the primary structure of the CM3 protein(s) is totally conserved in two species of *Triticum* and strongly suggests that CM proteins could play an important biological function (**17**). However, the *T.aestivum* UP2425 gene sequence shows changes in nt at two positions out of the coding region i.e. at +539 and at +574 positions, from CM3 cDNA clone pTd13.39 **(18)** while *T. durum* PDW 279 shows change at +574 position only. It can be due to mutation at that position. Change at +574 position can be neglected because it comes under reverse primer. While change at +539 position in UP2425 may be due to difference in cultivar used.

IV. FIGURES

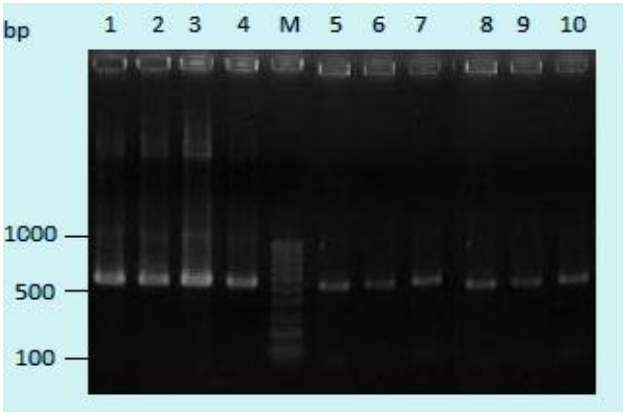

Fig.1 Agarose gel electrophoresis of purified 574 kb amplicon from different Indian wheat varieties. Lane M 100 bp Marker Lane 1-7 Kundan, Sonalika, UP1109, UP2003, UP2382, UP2425, UP262 genotype from species T. aestivum, and Lane 8-10 PDW 276, PDW274, PDW279 genotype from species T. durum

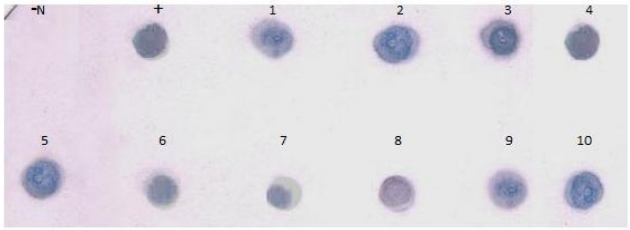

Fig. 2: Dot blot analysis of 0.57 kb amplicons from different varieties of T. aestivum and T. durum varieties to confirm the presence of gene. N- negative control, + Positive control, blot 1-7 Kundan, Sonalika, UP1109, UP2425, UP2003, UP2382, UP262 genotype from species T. aestivum, and blot 8-10 PDW 276, PDW274, PDW279 genotype from species T. durum

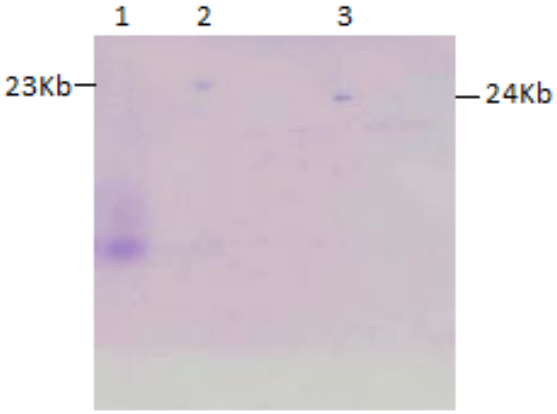

Fig. 3: Southern blotting analysis of plasmid from clone pTa2425 and pTd279 showing presence of gene.

Lane 1 Plasmid from pTd13.39 as positive control
Lane 2 Plasmid from pTd279
Lane 3 Plasmid from pTa 2425

Identify the constructs of a Journal – Essentially a journal consists of five major sections. The number of pages may vary depending upon the topic of research work but generally comprises up to 5 to 7 pages. These are:

```
1     ATGGCGTGCAAGTCCAGCTGCAGCCTCCTCCTCTTGGCCGCCGTCCTGCTCTCCGTCTTG
1      M A C K S S C S L L L L A A V L L S V L

61    GCCGCTGCTTCCGCCTCCGGCAGCTGCGTCCCAGGGGTGGCTTTTCGGACCAATCTTCTG
21     A A A S A S G S C V P G V A F R T N L L

121   CCACACTGCCGCGACTATGTGTTACAACAAACTTGTGGCACCTTCACCCCTGGGTCAAAG
41     P H C R D Y V L Q Q T C G T F T P G S K

181   TTACCCGAATGGATGACATCTGCGTCGATATACTCCCCTGGGAAACCGTACCTCGCCAAG
61     L P E W M T S A S I Y S P G K P Y L A K

241   TTGTATTGCTGCCAGGAGCTCGCAGAAATTTCTCAGCAGTGCCGGTGCGAGGCGCTGCGC
81     L Y C C Q E L A E I S Q Q C R C E A L R

301   TACTTCATAGCGTTGCCGGTACCGTCTCAGCCTGTGGACCCGAGGTCCGGCAATGTTGGT
101    Y F I A L P V P S Q P V D P R S G N V G

361   GAGAGCGGCCTCATCGATCTGCCCGGATGCCCCAGGGAGATGCAATGGGACTTCGTCAGA
121    E S G L I D L P G C P R E M Q W D F V R

421   TTACTCGTCGCCCCGGGGCAGTGCAACTTGGCGACCATTCACAATGTTCGATACTGCCCC
141    L L V A P G Q C N L A T I H N V R Y C P
                                  C
481   GCCGTGGAACAGCCTCTGTGGATCTAGAGATAAAATCAGTCGCTCGTGAATAAGCATGCA
161    A V E Q P L W I *

541   TGTTGCATCCATAGGCGTGTGGTGTGCATGTATGC
181
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Fig. 4: Nucleotide sequence and primary structure of wheat CM3 protein from pTa 2425 (Accession No. AY436554), and pTd 279 (Accession No. AY465898), clone. (shaded nucleotide shows change in sequence in pTd 279 which is C in place of G)

V. TABLES

Table-1: Comparisons of Wheat CM3 mRNA/ protein sequences (T.aestivum and T. durum) with different cereal alpha amylase/trypsin inhibitor source in terms of percentage identity. (-) is placed where no data is available.

Sl. No.	Description	Accession no.		Identity	
		mRNA	Protein	mRNA	Protein
1	Triticum aestivum alpha amylase inhibitor protein gene, complete cds CM3 protein	AY436554.1 X17574.1	P17314	100% 99%	100%
2	CMd component of tetrameric alpha-amylase inhibitor [Hordeum vulgare	X69939.1	P11643.2 CAA49536.1	86%	83% 81%
4	CMd3 protein [Hordeum vulgare]	U47640.1	AAB63440.1	87%	81%
5	Alpha-amylase/trypsin inhibitor CMb	X69938.1	P32936.2 CAA49556.1	64%	50%
6	Alpha-amylase/trypsin inhibitor CMa, component of tetrameric alpha-amylase inhibitor [Hordeum vulgare subsp. vulgare] BTAI1 mRNA encoding CMa,	X69937.1	P28041.2 CAA49555.1	65%	49%
7	Hordeum vulgare subsp. spontaneum itr2 gene for	FR746098.1	CAA72791.1	75%	38%

	barley trypsin inhibitor CMc precursor, cultivar Ardhawi				
8	trypsin inhibitor CMe precursor [H. vulgare]	-	CAA35188.1	-	39%
9	alpha-amylase inhibitor, tetrameric, chain CM16 precursor - durum wheat, Triticum aestivum	X55455.1	P16159.1, S08466, CAA35596.1C AA39100.1CA A34709.1	72%	51%
11	alpha amylase inhibitor CM1 [Triticum aestivum]	FN429984.1	P16850.1 CAA35598.1C AZ76052.1	75%	48% 44%
12	Alpha-amylase/trypsin inhibitor protein CM2 Triticum turgidum subsp. Durum, Triticum aestivum	FN435984.1	P16851.2 CAA39099.1 CBA13559.1	66%	49% 44%
14	CM 17 protein precursor [Triticum aestivum]	X59791.1	CAA42453.1	70%	44%
15	alpha amylase-trypsin bifunctional inhibitor [Eleusine coracana]	DQ494211.1	ABF47511.1	85%	37%
16	Zea mays subsp. parviglumis isolate hag_G7 Hageman factor inhibitor (hag) gene, complete cds	EU724313.1	ACJ62058.1	81%	44%
17	Dimeric alpha amylase inhibitor [Triticum aestivum]	-	BAA20139.1	-	27%
18	monomeric alpha amylase inhibitor [Triticum aestivum]	-	P01085	-	37%

Table-2: sequence alignment of different subunits of wheat tetrameric alpha amylase inhibitor protein (CM proteins) present in NCBI. Shaded areas are highly conserved sequences among CM protein subunits.

CM3 1MACKSSCSLLLLAAVLLSVLAAASASGSCVPGVAFRTNLLPHCRDYVLQQTCGTFTPGSKLPEWMTSASI70

CM16 MASKSNCVLLLAAVLVSIFAAVAAIGNEDCTPWMSTLITPLPSCRDYVEQQACRIETPGSPYLAKQQCCG

CM17 MASKSNYNLLFTALLVFIFAAVAAVGNEDCTPWTSTLITPLPSCRNYVEEQACRIEMPGPPYLAKQECCE

CM2 MASKSSITHLLLAAVLVSVFAAAAATGPYCYPGMGLPSNPLEGCREYVAQQTCGVGIVGSPVSTEPGNTP

CM1 MASKSSISPLLLATVLVSVFAAATATGPYCYAGMGLPINPLEGCREYVAQQTCGISISGSAVSTEPGNTP

CM3 71YSPGKPYLAKLYCCQELAEISQQCRCEALRYFIALPVPSQPVDPRSGNVGESGLIDLPGCPREMQWDFVR140

CM16 ELANIPQQCRCQALRYFMGPKSRPDQSGLMELPGCPREVQMDFVRILVTPGYCNLTTVHNTPYCLAMEES

CM17 QLANIPQQCRCQALRYFMGPKSRPDQSGLMELPGCPREVQMNFVPILVTPGYCNLTTVHNTPYCLGMEES

CM2 RDRCCKELYDASQHCRCEAVRYFIGRTSDPNSGVLKDLPGCPREPQRDFAKVLVTPGHCNVMTVHNTPYC

CM1 RDRCCKELYDASQHCRCEAVRYFIGRRSDPNSSVLKDLPGCPREPQRDFAKVLVTSGHCNVMTVHNAPYC

CM3 141LLVAPGQCNLATIHNVRYCPAVEQPLWI

CM16 QWS

CM17 QWS

CM2 LGLDI

CM1 LGLDI

VI. Conclusion

Wang et al (22, 23, and 24) reported that the populations of wild emmer wheat showed a wide range of diversity in WDAI, WMAI both between and within populations, he also found that alpha -amylase inhibitors are adaptively selected under different environments according to population and codon analysis.
However in the present study no significant changes in CM3 coding gene(s) have been observed within or between the species. It can be concluded that there are no effect of environmental or geological factor(s) on the CM3 coding gene. The conserved gene may play an important biological role which will perhaps be much more important than merely defense against pests.

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