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An *in silico* Analysis of Upstream Regulatory Modules (URMs) of Tapetum Specific Genes to Identify Regulatory *cis*-Elements and Transcription Factors

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Abstract

The present work presents an *in silico* analysis of Upstream Regulatory Modules (URMs) of genes expressed in tapetum specific manner in dicotyledon and monocotyledon plants. In the current analysis, we identified several motifs conserved in these URMs of which ten were observed to be part of known *cis*-elements using tools and databases like MEME, PLACE, MAST and TFSEARCH. We also identified that binding sites for two transcription factors, DOF and WRKY71 were found to be present in majority of the URMs.

Keywords

Tapetum Specific Promoter, *cis*-Elements, Transcription Factors

1. Introduction

Tapetum is the innermost layer of the anther wall of plants. It performs the function of a nourishing tissue that remains in continuity with the pollen mother cell through plasmadesmatal connections till the formation of meiocytes occurs in young anther. Tapetum varies from unilayer to multilayer in different plant species and can be uninucleate or multinucleate. Although tapetum cells form a single or at-most a few cell layers in the anther tissue, several studies have been carried out to understand how these cell layers develop and the functions played by them in pollen cell development [1] [2] [3] [4]. These studies have led to the identification of several genes expressed in tapetum specific manner. Such genes have mainly been identified by analyzing comparative cDNA libraries, subtractive hybridization, microarray analysis, *in-situ* hybridization and in recent years

by Laser Dissection Microscopy followed by RNA sequencing [1] [5]-[16].

TA29 from *Nicotiana tabacum* [1] and A9 from *Arabidopsis thaliana* [6] are examples of tapetum specific genes that were identified in early years. The promoters of these genes known as TA29 and A9 promoters have been used extensively in the expression of transgenes like *barnase* and *barstar* from *Bacillus amyloliquifaciens* to develop pollination control systems for hybrid seed production [5] [17] [18] [19] [20] [21]. Tight regulation of these promoters leading to tapetum specific expression was the key to success of this system. Attaining a robust tissue specificity of a promoter may need the combinatorial interplay of positive and negative regulators (transcription factors, TFs). The TFs would bring about their outcome by binding to the promoter through specific motifs or *cis*-elements. Several tapetum specific promoters have been identified till date, examples of which have been summarized in **Table 1**. However, there is limited knowledge about the transcription factors or the *cis*-elements of the promoters that are important for regulating these promoters. Although some tapetum specific promoters have been recently characterized in details e.g. OsLTP6 from rice [22] and A9 from *Arabidopsis* [23] in most of the studies, the characterization is limited to identifying the minimum length of the promoter needed for tapetum specific expression.

The present work is an attempt to identify conserved motifs/*cis*-elements present in genes expressing in the tapetum tissue of dicotyledon and monocotyledon plants. Further, putative TFs that may bind to these elements have also been predicted. Information generated from this work can be used for experimental validation.

2. Method

Motif Based Sequence Analysis Suite, MEME suite ver. 4.9.1 [24] was used to find out the conserved motifs in the different datasets. PLACE database [25] was used to figure out the *cis*-elements from the conserved motifs so obtained. Multiple Alignment & Search Tool, MAST ver. 4.9.1 [26] was used to attain consensus sequence of the conserved motifs obtained from MEME analysis. TFSEARCH software ver.1.3 [27] was used to find the putative TFBS and the transcription factors.

3. Results and Discussion

A literature survey was carried out to identify genes that expressed in a tapetum or anther specific manner. A total of 34 genes, 24 from dicot and 10 from monocot plants were identified and used in the present analysis (**Table 1**). From these, two datasets were developed comprising of 600 bp Upstream Regulatory Module (URM), one from dicot and another from monocot species. URMs [40] are defined as a region of a gene upstream to the translational start site, which includes the 5'UTR. Analyzing the URM was necessary in this analysis as in most cases the transcriptional start site has not been experimentally identified.

Table 1. Details of the URMs of the genes from both dicot and monocot plants used in the present analysis.

	S. No.	Gene name	Accession No. (Acc)/Gene ID (GI)	Plant	Reference
Dicots	1	*A*3 (Ata3)	GI: 3204096	*A. thaliana*	DS*
	2	*A*6 (Ata6)	GI: 22676	*A. thaliana*	[28]
	3	*A*9 (Ata9)	GI: 16143	*A. thaliana*	[6]
	4	*Ap*3 (Atap3)	GI: 940179	*A. thaliana*	[29]
	5	*Agp*1 (AtAgp1)	Acc: X68211	*A. thaliana*	[30]
	6	*Tap*2 (AmTap2)	Acc: X55434	*Antirrhinum majus*	DS
	7	*Tapnac*	GI: 842404	*A. thaliana*	[31]
	8	*Grp*18 (At5g07520)	GI: 830645	*A. thaliana*	DS
	9	*Bp*4*a* (BnBp4a)	Acc: X52874	*Brassica napus*	[32]
	10	*Bp*4*c* (BnBp4c)	Acc: X52874	*B. napus*	[32]
	11	*M*1 (Bnm1)	Acc: U86642	*B. napus*	DS
	12	*A*9 (Bja9)	AF134410	*B. juncea*	DS
	13	*Bgp*1 (BcBgp1)	Acc: X68210	*B. campestris*	[30]
	14	*TA*29 (NtTA29)	Acc: X52283	*Nicotiana tabacum*	[5]
	15	*Ntp*303 (NtNtp303)	Acc: X69440	*N. tabacum*	[33]
	16	*Ntm*19 (NtNtp19)	Acc: X88847	*N. tabacum*	[34]
	17	*A*37 (Nta37)	Acc: AY090039	*N. tabacum*	DS
	18	*Lat*52 (LeLat52)	Acc: 15855	*Lycopersicon esculentum*	[35]
	19	*Lat*56 (LeLat56)	Acc: X56487	*L. esculentum*	[36]
	20	*Lat*59 (LeLat59)	Acc: X56488	*L. esculentum*	[36]
	21	*TA*29 (LeTA29)	GI: AM261325	*L. esculentum*	DS
	22	Un_char protein/TA29-like	SolyC02g078370	*L. esculentum*	DS
	23	*TA*29 (StTA29)	GI: PGSC0003DMG400041062	*Solanum tuberosum*	DS
	24	*Taz*1 (PhTaz1)	Acc: AB063169	*Petunia hybrida*	[8]
Monocot	25	*OsRa*8	Acc: AF042275	*Oryza sativa*	[37]
	26	*Osg*6*B*	Acc: D21160	*O. sativa*	[38]
	27	*Osg*4*B*	Acc: D21159	*O. sativa*	[38]
	28	*OsRts*2	Acc: U12171	*O. sativa*	DS
	29	*E*1 (Ose1)	Acc: A23333	*O. sativa*	Patent: WO 9213956
	30	*T*42 (Ost42)	Acc: A23332	*O. sativa*	Patent: WO 9213956
	31	*T*72 (Ost72)	Acc: A23331	*O. sativa*	Patent: WO 9213956
	32	Af366296 (ZmAf366296)	Acc: AF366296	*Zea mays*	DS
	33	Af366294 (ZmAf366294)	Acc: AF366294	*Z. mays*	DS
	34	Af149016 (ZmAf366294)	Acc: AF149016	*Z. mays*	[39]

*DS—Direct submission.

The sequences for the respective URMs were downloaded from NCBI website. The sequence files of dicots and monocots URMs thus generated were submitted separately at MEME Tool available online for analysis of conserved motifs. In order to identify the conserved motifs, MEME program was run with different parameters that defined the motif width (5 - 13, 6 - 10 or 6 - 14) and the total number of motifs to be generated was fixed at 10. After identifying the motifs generated using the different widths, it was observed that in most cases, the motif generated with 6 - 14 width encompassed those generated by 5 - 13 or 6 - 10 width. Thus, the 10 motifs generated with 6 - 14 width were taken for further analysis. The position of the motifs in the different URMs as generated by MEME for both datasets and the sequence of the motifs as identified by MAST are presented in **Figure 1** and **Figure 2**.

After identifying the conserved motifs in the two datasets of anther/tapetum specific genes, the next step was to analyze if these motifs corresponded to any known *cis*-elements of plant promoters. This was done by creating strings of the identified motifs and submitting it to the PLACE database. This led to the identification of several known *cis*-elements. This data was then manually curated and 10 known *cis*-elements were identified that are enlisted in **Table 2**. It was observed that out of the 10 identified motifs in case of dicots (**Figure 1**), 6 of them have already been reported in the literature. In this case, no known *cis*-elements were identified for motifs 1, 2, 8 and 10. In case of monocots, we could identify known *cis*-elements only for motifs 1, 5 and 7. This could be reflective of the fact that generally more information is available for dicot promoters than those of monocots.

We then attempted to see if there was any information about TFs binding to these *cis*-elements. In order to do so, we first analyzed the presence of transcription factor binding sites using TFSEARCH tool. TFSEARCH searches highly correlated sequence fragments versus a TFMATRIX that is a transcription factor binding site profile database present in "TRANSFAC" database [27]. Strings of the motifs enlisted in **Figure 1** and **Figure 2** were submitted as query sequence to TFSEARCH which is available online. These led to the identification of four transcription factors which could bind to these URMs. These were DOF [45] and ICE1 [46] [47] [48] [49] [50] with predicted binding sites present in motif 7 and 5, respectively for monocot URMs. ICE1 was also found to have binding site in motif 7 of dicot URMs. Further, motif 7 in dicot URM was also a predicted binding site for the TF RAVI [54]. The TF, WRKY710S [55] was observed to have binding sites in URMs of dicots represented by motifs 3, 4 and 5.

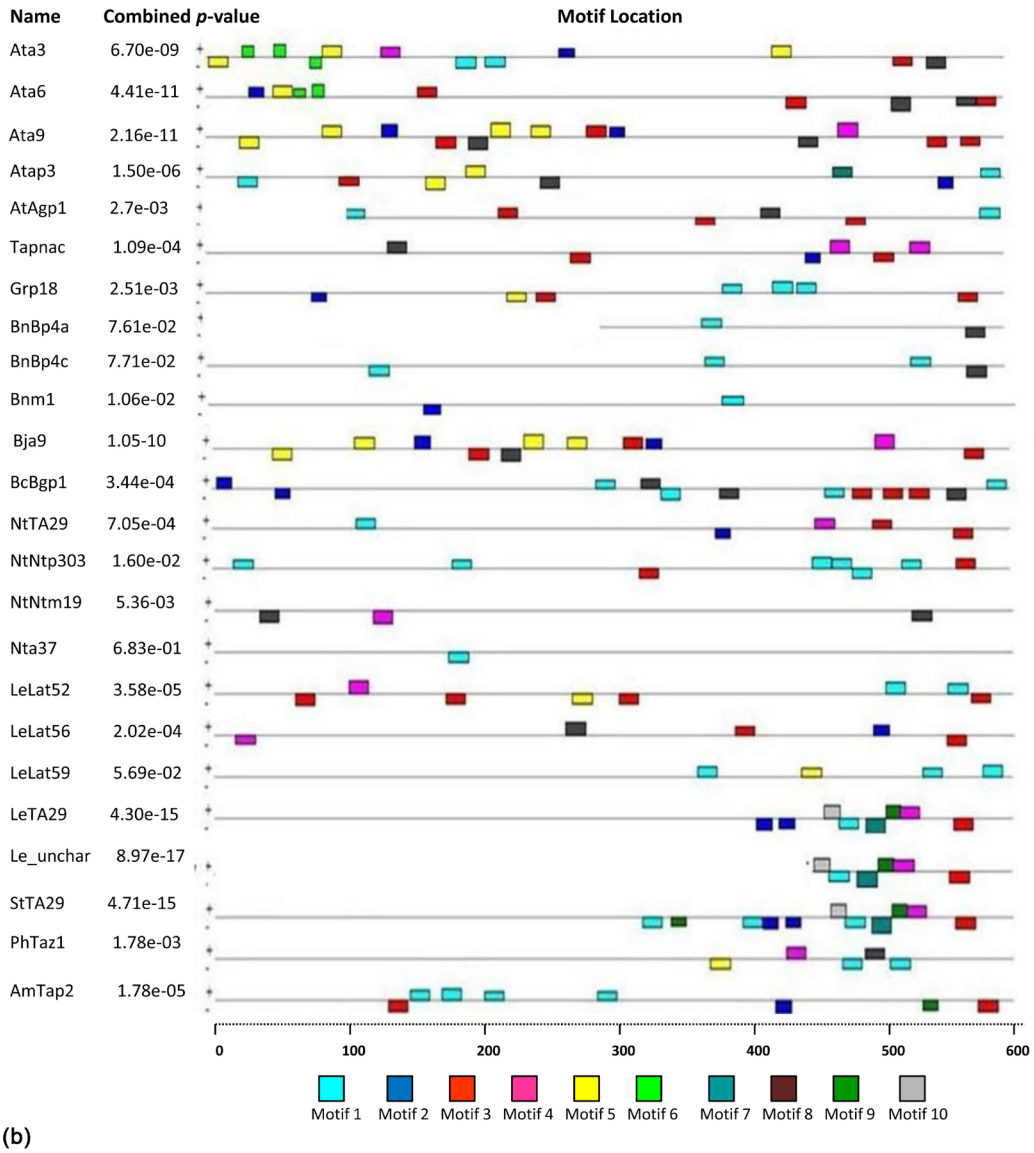
Of the 4 identified TFs, it was observed that only DOF and WRKY710S were found to have binding sites in majority of the URMs analyzed. The other 2 were represented in less than 20% of the analyzed URMs.

DOF stands for DNA binding with one finger domain proteins. They are a class of zinc finger transcription factors which are present in algae (*Chlamydomonas reinhardtii*) and moss (*Physcomitrella patens*) as single gene spreading

Table 2. Known *cis*-elements corresponding to identified motifs in **Figure 1** and **Figure 2** and transcription factors (TFs) binding to it, if any.

S. No.	Name of *cis*-element	Putative binding site	Function of the *cis*-element	TFs	Ref
1	ARR1AT	NGATT; N = A/T/G/C (Motif 5 & 6 of dicot)	ARR1 is a response regulator; found in *Arabidopsis*; N = G/A/C/T; AGATT is also present in the promoter of rice non-symbiotic haemoglobin-2 (NSHB) gene.	None	[41] [42]
2	BOX-4	ATTAAT (Motif 9 of dicot)	Part of a conserved DNA molecule involved in light responsiveness	None	[43]
3	CAAT-BOX	CAAT (Motif 7 of dicot)	"CAAT promoter consensus sequence" found in *legA* gene of pea	None	[44]
4	DOFCOREZM	AAAG (Motif 7 of monocot)	Core dof protein binding site was found in maize; zinc finger, and is unique to plants; Four cDNAs encoding Dof proteins, Dof1, Dof2, Dof3 and PBF, have been isolated maize; PBF is an endosperm specific Dof protein that binds to form prolamin box; and enhances transcription from the promoters of both cytosolic orthophosphate promoter and non-photosynthetic *PEPC* gene	DOF	[45]
5	E-BOXNNAPA/ MYCCONSENSUAT	CANNTG; N = A/T/G/C (Motif 7 of dicot) (Motif 5 of monocot)	The site is present in the promoter of *rd*22 (dehydration-responsive gene) and in many other genes in *Arabidopsis*; Binding site of ATMYC2 (previously known as rd22BP1); N = A/T/G/C; MYC recognition sequence in CBF3 promoter; Binding site of ICE1 (inducer of CBF expression 1) that regulates the transcription of CBF/DREB1 genes in the cold in *Arabidopsis*. E-box of napA storage-protein gene of *Brassica napus*. This sequence is also known as RRE (R response element).	ICE1	[46] [47] [48] [49] [50]
6	GTGANTG10	GTGA (Motif 5 of dicot)	"GTGA motif" found in the promoter of the tobacco late pollen gene g10 which shows homology to pectate lyase and is the putative homologue of the tomato gene lat56; Located between −96 and −93	None	[51]
7	POLLENLELAT52	AGAAA (Motif 1 of monocot)	Regulatory element responsible for pollen specific activation of tomato lat52 gene; Found at −72 to −68 region. Also found in the promoter of tomato endo-beta-mannanase gene.	None	[52] [53]
8	RAV1AAT	CAACA (Motif 7 of dicot)	RAV1 binding consensus sequence, binds to DNA with bipartite sequence motifs of RAV1-A (CAACA) and RAV1-B (CACCTG); RAV1 protein contains AP2-like and B3-like domains which recognize the CAACA and CACCTG motifs, respectively; The expression level of RAV1 is relatively high in rosette leaves and roots in *Arabidopsis*	RAV1	[54]
9	WRKY710S	TGAC (Motif 3, 4 & 5 of dicot)	A core of TGAC-containing W-box is present in *Amy*32*b* promoter; is binding site of rice WRKY71, which acts a transcriptional repressor in the gibberellin signaling pathway; Parsley WRKY proteins bind specifically to TGAC-containing W box elements within the Pathogenesis-Related Class10 (PR-10) genes	WRKY71	[55]
10	10PEHVPSBD	TATTC (Motif 4 of dicot)	"−10 promoter element" present in *psbD* gene promoter of barley; is required for expression of the *psbD* (plastid gene) encoding chlorophyll-binding protein of photosystem II reaction center, activated by blue/UV-A light/white light.	None	[56]

(a)

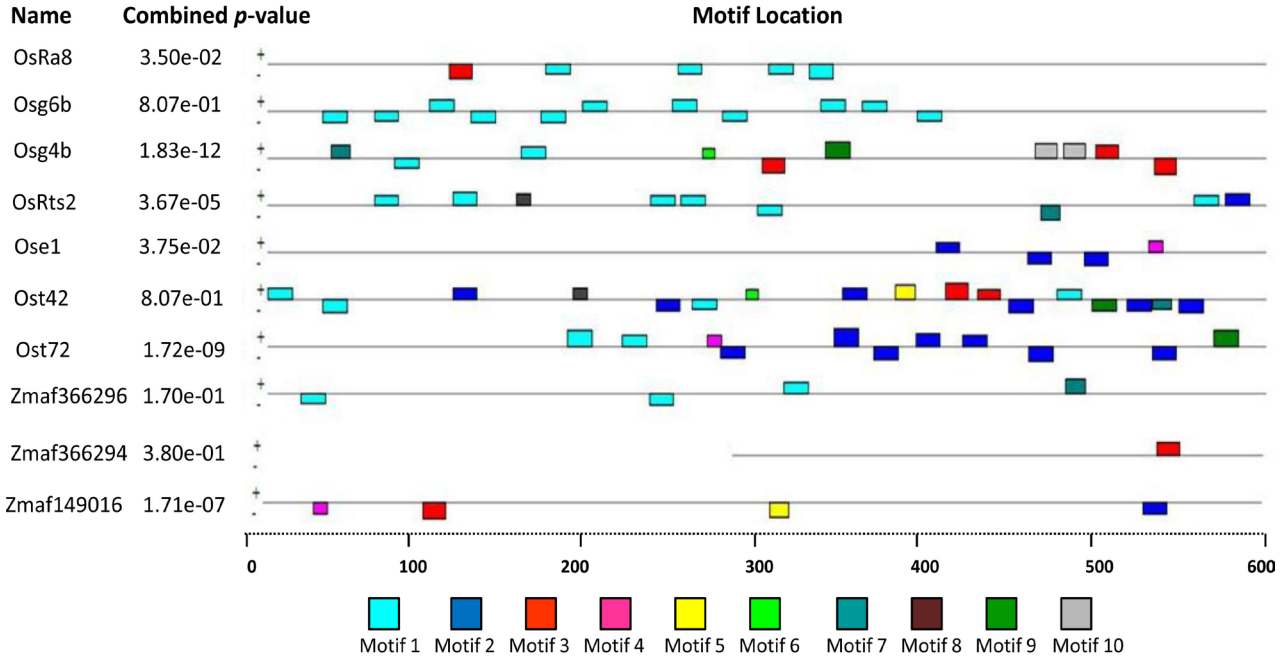


(b)

Motif No.	Consensus sequence	% representation in the dataset	Motif No.	Consensus sequence	% representation in the dataset
1	AAGAAGAAGGAGAA	75	6	CCGCCGGCG	8
2	TGGTGGGAGCG	58	7	AACACAATTGGAGC	13
3	GTTTTGGTGGGTGA	71	8	TGGTTTGTTTTGG	54
4	CCTATTTATTCTC	50	9	GGAACTTGTGG	17
5	GGTCACCCTCGAA	38	10	GTGCCTCAGA	13

Figure 1. Analysis of conserved motifs in URM (600 bp) of anther/tapetum specific genes of dicots using MEME tool. (a) Placement of the motifs in the URMs and (b) the consensus sequence of the various motifs obtained from MAST analysis. The presence of each motif in different promoters is mentioned as % representation.

(a)



(b)

Motif No.	Consensus sequence	% representation in the dataset	Motif No.	Consensus sequence	% representation in the dataset
1	AAAAAAAAAGAAA	70	6	CGGGGCC	20
2	GGCGCGCGCGCGGTG	50	7	GTTGGGGGGTC	40
3	GCCGCTGCTGCTT	50	8	CCACGCCC	20
4	CGCCGGCC	30	9	GAGCCCCACGGCGG	30
5	CCCACGTGCCG	20	10	CCGGCCGCCCCC	10

Figure 2. Analysis of conserved motifs in URM (600 bp) of anther/tapetum specific genes of monocots using MEME tool. (a) Placement of the motifs in the URMs and (b) the consensus sequence of the various motifs obtained from MAST analysis. The presence of each motif in different promoters is mentioned as % representation.

across lower and higher plants possessing multiple genes encoding the Dof domain containing protein [57]. Its cDNA was first isolated from maize [58] and in higher plants the Dof domain containing proteins have been identified in *Arabidopsis*, tomato, potato, pumpkin, pea, wheat, rice and barley [59]. The Dof domain is involved in both protein-protein interaction and acts as a DNA binding domain. The sequence specific binding of the Dof domain to AAAG motif has been verified by both in vivo and in vitro experiments [60] [61]. The Dof domain proteins are known to be involved in biological processes like seed germination, development and plant defense and light responses where they act as

both activator and repressors [59] [62]. It is involved in regulation of genes of specific pathway for carbon metabolism in maize where it regulates C4PEPC (C4 photosynthetic phosphoenol-pyruvate carboxlase), cyPPDK (cytosolic pyruvate orthophosphate dikinase) and non photosynthetic PEPC [45].

WRKY71 belongs to WRKY family of transcription factors. They are reported to be present across lower eukaryotes (protista) to ferns (pteridophytes) and in plants [63]. The WRKY family members are identified by the presence of a conserved 60 amino acid residue region and a zinc finger domain. Promoters of genes carrying the W-box are potential targets of the WRKY factors [55]. They are key components in the innate immunity of the plant and bind to the W-box of pathogenesis related genes [55] [64]. They are involved in seed and trichome development and embryogenesis [63]. They function as both activators and repressors by protein-protein interaction and autoregulation [65]. WRKY71 expresses in the aleurone layer in rice and is reported to function as a repressor of gibberellic acid signalling pathway in aleurone layer cells. GA pathway is involved in growth and development of plants [66].

The present analysis has led to the identification of certain elements and TFs that could regulate tapetum specific promoters. However, the role of these needs to be experimentally analysed. This can be done by a "loss-of-function" strategy in which the cis-elements in a given URM are mutated and changes in promoter activity, if any are analysed. In a second strategy, "gain-of function", a given TF can be ectopically expressed and its influence on the activity of a given URM is recorded.

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