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Isolation and Sequence Analysis of Wheat Tissue-Specific cDNAs by Differential Display

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Abstract The differential display technology was used to study and isolate tissue-specific cDNA from wheat (*Triticum aestivum*). cDNA was synthesized by reverse transcription from total RNA from wheat leaves, anthers, and ovaries to search for and isolate tissue-specific cDNAs and use them to screen wheat genomic library to get the corresponding genomic DNA clone. Here, we report the isolation, cloning, and sequencing of various tissue-specific cDNA fragments. Further, we report the isolation of a wheat genomic clone, 18-3. The clone has an unknown open reading frame (ORF238) that is similar to related grain EST sequences, 1,673 bp 5′ flanking region from the ATG and 1321 3′ flanking region. A PlantCARE database search using the 5′ flanking region revealed that there are many *cis*-acting elements in this region. About 109 *cis*-acting elements from different plant gene promoters in 24 groups were detected. For example, 26 CAAT elements, a common *cis*-acting element in promoter and enhancer regions, were detected overall the 5′ flanking region. Multiple TATA-boxes concentrated in three spots and a putative transcription start site also were detected. In addition, MeJA, DRE, GC-motif, ABRE, GCN4_motif, Skn_1-motif, HSE, A-box, ACE, G-box, I-box, TCCC-motif, P-box, TATC-box, and WUN-motif were detected. The putative function of the reported ORF238 and its promoter is unknown.

Keywords Wheat · Anther-specific expression · Promoter · *cis*-acting elements · Differential display

Introduction

Plants respond to biotic, abiotic, and developmental signals by the spatial and temporal regulation of gene expression. During plant development, gene expression diverges from the original set of genes expressed in the young seedling tissues through an overlap transition in the gene expression profile in various organs and tissues. This is achieved by the regulation of gene expression through a complicated combination of *cis*-acting elements in gene promoters and their corresponding *trans*-acting factors. Precise functional analysis of *cis*-acting elements and their binding transcriptional factors would lead to more understanding of the mechanism by which the expression of these genes leads to specification and organ development as well as plant response to various stresses and signals. This may lead to better exploitation of this information in the recent plant biotechnology to improve plant production and quality. Studying tissue-specific expression is an essential step toward the isolation and characterization of tissue-specific promoters.

Many plant promoters have been isolated and characterized including various tissue-specific promoters. For example, seed storage protein promoters have been characterized from wheat (Robert et al. 1989; Thomas and Flavell 1990; Aryan et al. 1991; Lamacchia et al. 2001), rice (Yoshihara et al. 1996; Washida et al. 1999), bean (Bustos et al. 1991), brassica (Ellerstrom et al. 1996), pea (Shirsat et al. 1989),

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and maize (Cao et al. 2007). In addition, some pollen-specific promoters have been isolated and studied in Zmg13 gene of maize (Guerrero et al. 1990), G9 gene of cotton (John and Petersen 1994), and lat52 gene of tomato (Bate and Twell 1998). Moreover, gene promoters responding to abiotic stresses have been studied extensively including osmotic- and cold-responsive promoters (Yamaguchi-Shinozaki and Shinozaki 2005; Zhu 2002; Baker et al. 1994; Stockinger et al. 1997; Yamaguchi-Shinozaki and Shinozaki 1994) and light-responsive promoters (Donald and Cashmore 1990; Puente et al. 1996; Lu et al. 1998; Martinez-Hernandez et al. 2002).

Two approaches have been used for promoter structure/function analysis to establish consensus of *cis*-acting elements in different promoters: first, the deconstructive approach which include 5′ or 3′ promoter deletion, deletion scanning, and changing *cis*-acting elements. Using this approach led to the detection of various common *cis*-acting elements and their *trans*-acting factors in stress-inducible promoters (Liu et al. 1994; Yamaguchi-Shinozaki and Shinozaki 1994, 2005); second, the reconstructive approach in which a synthetic promoter is constructed through combining functional *cis*-acting elements or large domains of element combinations that can be functionally tested (Venter 2007; Cazzonelli and Velten 2008). Such approach has provided valuable information about combining large upstream domains with different core regulatory regions (Odell et al. 1985; Ni et al. 1995; Li et al. 2004). Both of the deconstructive and reconstructive approaches provide new insights into the area of discovering new *cis*-acting elements and their cognate *trans*-acting factors.

Characterization of plant promoters revealed many different *cis*-acting elements. Some of these elements are basic promoter elements such as TATA-box and CAAT-box, yet others may be required for tissue-specific regulation or stimulus responsiveness. A combination of different *cis*-acting elements is essential to confer tissue specificity or responsiveness to stimuli. CAAT-box is a common *cis*-acting element in plant gene promoter. Osmotic- and cold-responsive elements have been detected and studied in a set of gene promoters (reviewed by Yamaguchi-Shinozaki and Shinozaki 2005), for example, abscisic acid responsive element (ABRE) in the Em gene (Zhu 2002) and CRT (GGCCGACAT) in the promoter of Cor15A gene (Baker et al. 1994; Stockinger et al. 1997). Dehydration-responsive elements, such as DRE (TACCGACAT), in the promoter of RD29A gene were analyzed (Yamaguchi-Shinozaki and Shinozaki 1994). Light-responsive elements have been characterized in many nuclear-encoded photosynthetic genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase and genes encoding chlorophyll a/b binding proteins (CAB). I-box, G-box, and GT-box are three conserved elements in the promoter regions of many light-regulated genes (Donald and Cashmore 1990). A conserved modular arrangement (CMA5) was found to be the minimal light-responsive unit for light responsiveness (Arguello-Astorga and Herrera-Estrella 1996) in many plant light-responsive promoters. More analysis of CMA5 module revealed that the S-box is for sugar and ABA responses (Acevedo-Hernandez et al. 2005). Small stretches of purine and pyrimidine, RY repeats, with potential to form Z-DNA have been reported in gliadin promoters (Aryan et al. 1991). A sequence of 45 bp that contains AACA and GCN4 motif confirmed endosperm-specific expression of glutelin, *GluA-3*, in rice (Yoshihara et al. 1996).

Determination of specific function of different *cis*-acting elements in different plant promoters is a difficult task because each promoter has its unique organization of *cis*-acting elements and consequently ability to furnish a platform for binding of *trans*-acting factors. This depends on *cis*-acting elements number, spacing, arrangement, orientation, and their super hierarchal order in large domains (Arguello-Astorga and Herrera-Estrella 1996; Acevedo-Hernandez et al. 2005; Cazzonelli and Velten 2008). It is accepted that these combinations of *cis*-acting with the presence or absence of different *trans*-acting factors in different plant tissues and/or species orchestrate the symphony of tissue- or species-specific expression of plant promoters. This support the idea that additional experimental studies are required to determine the function of individual *cis*-acting elements in plant promoters, discover new elements, and study how *cis*-acting elements with their respective *trans*-acting factors work together to regulate transcription in a tissue-specific manner. Well-characterized plant promoters are needed because (1) the number of well-studied promoters is low compared to the number of different messages expressed in different tissues, (2) different promoters in different plants are independent cases since a promoter could be tissue-specific in one plant or one tissue and is not in another, and (3) tissue-specific promoters are needed now more than ever for use in plant biotechnology applications to limit the targeted gene expression to specific tissues for the production of plants with certain qualities like pest resistance and production of specific proteins in limited tissues. Therefore, the isolation and characterization of more tissue specific-promoters is an interesting field for more studies both at the basic and applied science levels.

Here, we report the isolation, cloning, and sequence analysis of many cDNA fragments of wheat tissues using differential display (DD) procedures. Sequence analysis of genomic clone isolated from wheat anthers by anther-specific fragment revealed many different *cis*-acting elements arranged in super hierarchal order that could be responsible for the expression from a putative promoter in response to different stimuli.

Materials and Methods

Isolation of Total RNA from Wheat Tissues

Total RNA was isolated from wheat (*Triticum aestivum* cs) leaves, anthers, and ovaries using TriReagent solution (MRC). DNA contamination was removed from RNA preparations using the MessageClean Kit (GenHunter). The final pellet was dissolved in DEPC-treated water. Concentrations of RNA were determined at A_{260} and RNA samples were stored in aliquots at −85°C.

Differential Display Procedures

RNAimage kits (GenHunter) were used to prepare the differentially expressed mRNA (Liang and Pardee 1992) as ^{33}P-labeled cDNA fragments following manufacturer instructions. Polymerase chain reaction (PCR) was done using the first-strand cDNA as template and the same anchoring primer (polyT) in combination with various arbitrary (random) primers. Labeled cDNA PCR fragments, 3.5 μL, were separated on 6% denaturing sequencing gel. Samples were electrophorized for 3.5 h at 60 W constant power. After electrophoresis, the gel was blotted onto 3MM paper and dried under vacuum for 1 h at 80°C. The dried gel was marked with radioactive ink and exposed to X-ray film for 24 to 72 h.

Recovery and Reamplification of cDNA Fragments

The autoradiogram and the dried gel were aligned using the radioactive ink marks. Bands of interest were located, cut out with a clean razor blade, soaked for 10 min in 100 μL of deionized water, and boiled for 15 min. Tubes were centrifuged for 2 min and the supernatant was transferred to a new tube. cDNA was recovered by ethanol precipitation and dissolved in 10 μL of deionized water. Recovered cDNA, 4 μL, was used for reamplification by PCR under the same conditions except that the dNTPs concentration was at 20 μM instead of 2 μM and no isotope was included. PCR products, 20 μL, were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. DNA bands of interest were cut from agarose gels and stored at −20°C. DNA fragments were recovered from gel slices using GenElute agarose spin columns (Supplco). Recovered DNA fragments were ligated into pGM-T vector (Promega) and transformed into *E. coli* XL1-Blue competent cells.

Reverse Northern Blotting

Colony Lifts

Ten cloned DD fragments for each tissue were selected and used in reverse Northern blotting (Zegzouti et al. 1997). Five white colonies for each selected fragment were inoculated onto a 15-cm LB/Amp plate in a certain order. Colonies were allowed to grow to a suitable size and three lifts were prepared from the plate onto nitrocellulose membranes. All lifts were treated on 3MM filter paper saturated with the indicated solution: 10 min on 0.5 N NaOH, 5 min on 1 M Tris, pH 8.0, 5 min on 1 M Tris, pH 8.0, 1.5 M NaCl. Colony lifts were washed twice for 5 min in 2X SSC and allowed to air dry. Lifts were baked for 20 min at 80°C and stored at room temperature.

cDNA Probe Synthesis

Total cDNA probe from different wheat tissues was prepared using a published method with some modifications (Zhang et al. 1996). Total RNA from leaves, anthers, or ovaries, 10 μg, was used to prepare cDNA probe using the reverse transcription (RT) reaction. The reaction was assembled in RT buffer (GenHunter), containing 30 μM dNTPs (-dCTP), 0.8 μM of T20 primer, 1.67 μCi/μL of [α-^{33}P]dCTP, and 500 U of MMLV reverse transcriptase. Before adding, the enzyme tubes were incubated in the thermocycler for 5 min at 65°C. After 10 min at 37°C, the reverse transcriptase was added and incubation was continued for 50 min at 37°C. The PCR program was: 5 min at 65°C, 1 h at 37°C, and 5 min at 72°C. RT-PCR products were ethanol-precipitated, washed, dried, and dissolved in H_2O. Dilutions, 1/10, 1/10^2, and 1/10^3, were prepared from each probe, and radioactivity was determined by scintillation counting.

Hybridization

Membranes for the three colony lifts were hybridized to the three cDNA probes. The membranes were prehybridized for 1 h in 20 mL of hybridization buffer (6X SSPE, pH 7.0, 5X Denhardt's reagent, 0.5% SDS) at 65°C. The buffer was removed and the probe (30×10^6 cpm) was boiled for 10 min and kept on ice. The denatured probe was mixed with 2.5 mL of hybridization buffer and added to the corresponding membrane in a hybridization bottle. Hybridization was done for 15 h at 65°C with rotation in a Techne Hybridizer HB-1 chamber. The membranes were washed twice in 2X SSC with 0.1% SDS at room temperature and twice in 0.5X SSC with 0.1% SDS at 65°C. The washed membranes were exposed to X-ray film for 1 to 7 days.

Northern Blotting

Total RNA, 20 μg, of wheat tissues (leaves, anthers, ovaries) were electrophoresed in 1% agarose gels in 0.5X TBE running buffer for 1.5 h. RNA was transferred to Nylon membrane using a downward blotting protocol

(Zhou et al. 1994). DD-specific ^{32}P-labeled DNA probes were synthesized using Prime-a-Gene kit (Promega). Hybridization and washing were carried out as explained in the "Genomic Library Screening" section below.

Plasmid Isolation and DNA Sequencing

White colonies, three to five for each fragment, were grown overnight in LB media in 96-well plates for plasmid isolation and sequencing at the DNA Sequencing and Genotyping Facility (Kansas State University) using T7 or T3 primers.

Database Search

DNA sequencing data were trimmed to remove excess vector sequences and used to search the database using the BLAST engine at the National Center for Biotechnology Information site (Altschul et al. 1997)

Genomic Library Screening

Wheat genomic library λsep6-lac5 donated by Dr. Olin Anderson (USDA, ARS, Albany, CA), 1.5×10^6 pfu/μl, was screened with the 18-1 DNA probe. DNA probe was prepared using Prime-a-Gene kit (Promega) according to the manufacturer's instructions using a PCR-amplified 18-1 DNA fragment. Double lifts from the genomic library plates, 5×10^4 pfu/plate, were prepared on nylon membrane filters following standard protocol (Sambrook et al. 1989). Filters were blocked for 1 h in hybridization buffer, 5X SSC, 0.1% *N*-lauroyl-sarcosine, 0.02% SDS, 1% blocking reagent (Roche), at 65°C. Denatured DNA probe was added in 5 mL of the hybridization buffer and allowed to hybridize overnight. Filters were washed twice in 2XSSC, 0.5% SDS for 30 min at room temperature and twice in 2XSSC, 0.1% SDS at 65°C. Washed filters were exposed to X-ray films for 12–24 h at −80°C. Plaques isolated from the first screening went through a second round of screening.

Genomic DNA Sequencing and Analysis

DNA from the genomic clone 18-3 was purified using Wizard Lamda Preps DNA Purification System (Promega). The isolated DNA was sequenced using primer walking approach starting with DD reverse primer. This offers the possibility of getting the sequence from 3′ end toward the 5′ end of the gene. Sequences were aligned and used for a primary database search (BLAST) and ORF search (ORF finder, NCBI). A 5′ primer was designed to amplify the sequenced 3.711-kb fragment using PCR. The fragment was cloned in pGM-T vector and transformed into XL1-blue competent cells. One white clone, 18-3, was used for plasmid isolation and the insert was sequenced one more time. The final results of sequencing were used for database and ORF searches. The 5′ flanking region of a detected ORF (ORF238) was used to search the PlantCARE database (plant *cis*-*a*cting *r*egulatory *e*lements) for *cis*-acting promoter elements (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; Lexcot et al. 2002).

Results

Differential display was used to study differential gene expression and isolate tissue-specific cDNAs for the further isolation of tissue-specific promoters from wheat leaves, anthers, and ovaries. Using RNAimage kits (GeneHunter), 60 cDNA fragments were recovered from various DD gels: 18 for leaves, 32 for anthers, and ten for ovaries. The selected fragments showed a differential presence on DD gels in duplicate lanes. Examples of DD fragments are shown in Fig. 1. The indicated fragments are detected in the duplicate lanes, eliminating a high portion of false positives. For example, fragment AF18-1, AF22, and LF59 are clearly differentially expressed. The recovered fragments range in size from 200 to 600 bp (Table 1). All of

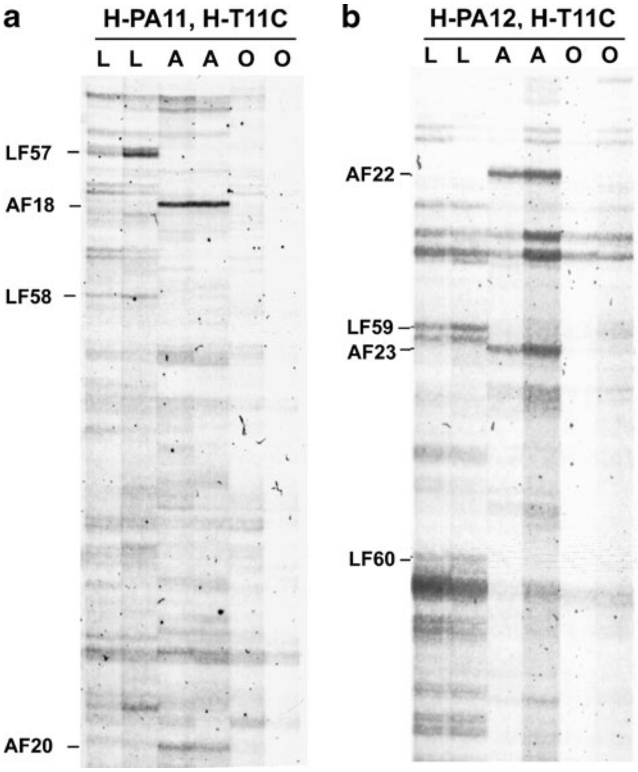

Fig. 1 Representative regions of differential display gels showing DD fragments amplified with two combinations of anchoring (H-T11C, polyT) and arbitrary (random) primers. **a** H-PA11, H-T11C primers. **b** H-PA12, H-T11C primers. *AF*, anther fragment; *OF*, ovary fragment; *LF*, leaf fragment

Table 1 DD fragments that showed good sequences and/or database hits

Fragment	Size, bp	Clone	Database similarities / (organ or tissue for ESTs)	*I* (%)	Gene Bank ACC #	Organism, database
AF-3	440	AF3-1	Post-anathesis spike	100	BQ236843	TA, EST
AF-7	204	AF7-3				
AF-8	494	AF8-2	Spike at flowering date	84	BJ247975	TA, EST
		AF8-3	Spike before anathesis	98	BQ170899	TA, EST
AF9	375	AF9-3	Fibers	37	BQ413432	GA, EST
AF-10	284	AF10-1	Spike at flowering date	96	BJ254501	TA, EST
		AF10-2	Germinating seeds	75	BQ465420	HV, EST
AF-11	347	AF11-1	Spike at flowering date	99	BJ248350	TA, EST
		AF11-3	Spike at flowering date	98	BJ245780	TA, EST
AF-18	621	AF18-1	Spike before anathesis	69	BQ170375	TA, EST
AF-19	393	AF19-1	Spike at flowering date	99	BJ318301	TA, EST
		AF19-2	Spike before anathesis	93	BQ169388	TA, EST
		AF19-3	Spike at early flowering	94	BJ322244	TA, EST
AF-20	365	AF20-1	NADH dehydrogenase	90	CAB59284	HV, nr
AF-21	505	AF21-1	MAP Kinase 9	47	BAA92223	AT, nr
AF-22	510	AF22-1	Rice sperm (pollen)	73	BE225318	OS, EST
AF-23	438	AF23-3	Isocitrate dehydrogenase	88	BAA88179	OS, EST
			Wheat *Fusarium*-infected spike	96	BE585970	TA, EST
AF-29	451	AF29-3	Isocitrate dehydrogenase	87	BAA88179	OS, EST
			Seeds	93	BJ238301	TA, EST
AF-30	428	AF30-3	Rice sperm (pollen)	73	BE225318	OS, EST
OF-40	179	OF40-2	Pre-fertilization ovules	96	AL830276	TA, EST
LF-43	204	LF43-2	Normal seedlings	96	AL827128	TA, EST
LF-45	611	LF45-1	Putative protein	35	CAB66408	AT, nr
LF-46	408	LF46-1	Putative protein	85	CAB45319	AT, nr
		LF46-3	HMBS	64	CAA51820	PS, nr
LF-47	274	LF47-1	Leaves	82	BG906715	TA, EST
LF-48	366	LF48-1	Drought stressed leaves	65	BF478390	TA, EST
LF-49	310	LF49-3	Callus	50	D22919	OS, EST
LF-50	194	LF50-2	Spike at early flowering	76	BJ318521	TA, EST
LF-53	606	LF53-1	Putative protein	88	CAB78385	AT, nr
LF-54	605	LF54-1	Putative protein	87	AL049608	AT, nr
LF-57	661	LF57-3	Immature leaf primordium	89	BI245242	ZM, EST
LF-59	452	LF59-1	Chloroplast ATP synthase beta, epsilon subunits	100	WHTCPATPB	TA, nr

HMBS hydroxymethylsbilaine synthase, *AT Arabidopsis thaliana*, *HV Hordum valgure*, *TA Triticum aestivum*, *OS Oryza sativa*, *PS Pisum sativum*, *CP* chloroplast, *AF* anther fragment, *OF* ovary fragment, *LF* leaf fragment, *I* identity, *AC* accession number, *GA Gossypium arboreum*, *ZM Zea mays* (cloned differential display fragments with sequences that have similarities to databases sequence)

the recovered fragments were successfully amplified using the same primer set and PCR conditions. A total of 46 fragments, 17 for leaves, 19 for anthers, and 10 for ovaries, were cloned in pGM-T vector (Promega). Selection for cloning was based on size and appearance on DD gels (Table 1).

Reverse Northern blotting is one of the tools available to early screen DD fragments by their response to the total cDNA probes from tissues under study. It was used for preliminary confirmation of tissue specificity for some of the cloned fragments using the total cDNA probes from the three tissues for hybridization to the cloned cDNA. Five white colonies of each tested fragment were used in reverse Northern blots. Fragments AF18 and AF22 are two examples of anther fragments that showed differential response to the cDNA probes (Fig. 2). Three clones (1, 2, 5) of AF18 fragment and five clones (1–5) of AF22 fragment gave hybridization signals with cDNA probe from anthers only, whereas the same clones gave no response to cDNA probes from leaves and ovaries. This indicates that these fragments could represent anther-specific messages; however, some fragments do not respond to any of the three probes.

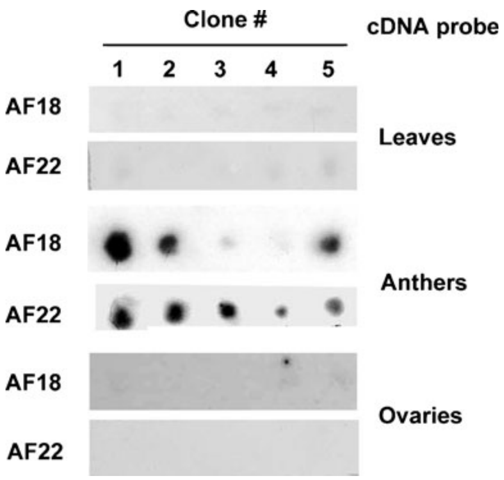

Fig. 2 Reverse Northern blot demonstrating the response of five representative clones of anther fragments AF18 and AF22 to total cDNA probes. *AF*, anther fragment

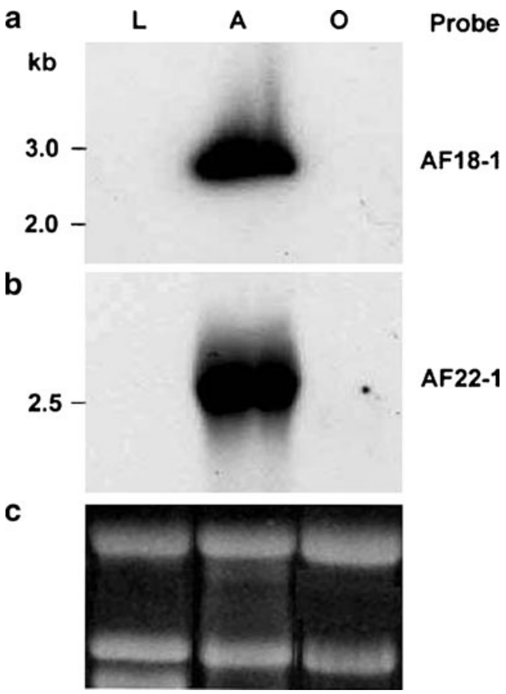

Fig. 3 Northern blot analysis of wheat total RNA of leaves (*L*), anthers (*A*), and ovaries (*O*) probed with AF18- and AF22-specific DNA probes. **a** Total RNA probed with AF18-specific DNA probe. **b** Total RNA probed with AF22-specific DNA probe. **c** rRNA of the agarose gel used on Northern blot to show equal loading. Lower extra band in leaves lane is a chloroplast rRNA

Sequencing and Database Search

Insert DNA from five colonies were sequenced for each cloned fragment. Some fragments contained more than one cDNAs while the rest of them show that each recovered fragment has only one cDNA. Most of the recovered sequences of the cloned DD fragments show similarities to known protein sequences, putative protein sequences, or EST sequences, whereas a few of them show novel cDNAs (Table 1). Clones 46-1 and 46-3 have cDNAs that correspond to a putative protein from *Arabidopsis thaliana* and pea hydroxymethylbialane synthase (HMBS), respectively. Three fragments, AF21, AF22, and AF30, show similarities to the same database sequence (ATMAPK9, AB038694) and two other fragments, AF23 and AF29, show similarities to isocitrate dehydrogenase. Some other fragments, such as LF45 and LF46-1 (Table 1), show similarities to putative proteins. Many fragments show similarities to EST sequences from similar tissues (Table 1). AF18 fragment shows similarity to an EST sequence from wheat spike before anathesis. This is further evidence that this fragment is anther specific.

Northern Blotting

Northern blotting was used to confirm the tissue specificity of two selected anther fragments, AF18 and AF22. A Northern blot for total RNA of leaves, anthers, and ovaries was prepared and probed by AF18- or AF22-specific DNA probe. Only anther RNA responded to both of these probes, whereas leaves and ovaries RNA gave no detectable signals. The detected 2–3 kb band in both cases gives strong evidence that their expression as RNA is anther specific (Fig. 3).

Wheat Genomic Library Screening

We selected fragment AF18-1 to screen wheat genomic library to isolate the promoter which control the expression of AF18 in wheat anthers. Three genomic clones were isolated: 18-3, 18-5, 18-16. Clone 18-3 which has an insert of 11 kb was used for sequencing by primer walking. Sequencing of 3.7 kb upstream of AF18 reverse primer showed that this sequence has a 717-bp open reading frame (detected by ORF finder, NCBI) which code for unknown protein (ORF238). The sequence has a 1,321-bp 3′ flanking region and 1,673-bp 5′ flanking region.

The 1,673-bp upstream of the ORF238 was used to search the PlantCARE database. This search resulted in the detection of 24 various *cis*-acting elements from plant gene promoters (Table 2; Fig. 4). Each of these *cis*-acting elements is repeated 1–26 times (Table 2). The highest is the CAAT-box with 26 time representation from eight different plant species. CAAT-box is a common *cis*-acting element in promoter and enhancer regions. TATA-box was detected nine times from six different plant species (Keddie et al. 1992; Coca et al. 1996; Carranco et al. 1997; Prieto-Dapena et al. 1999; Arguello-Astorga and Herrera-Estrella 1996; Manjunath and Sachs 1997; Pasquali et al. 1999; Dasgupta et al. 1993; Pastuglia et al. 1997). A putative

Table 2 *cis*-acting elements in 183 5′ flanking region

Function	PlantCARE	Sequence	Positions from ATG	Organism	Reference
Common *cis*-acting element in promoter and enhancer regions	CAAT-box	CAAT	−1549	NT	Klotz and Lagrimini 1996
		tCCAAa	−1238	AT	Caubet et al. 1996; Hillebrand et al. 1996; Vauterin et al. 1999
		gCCAAc	−1019	AT	Caubet et al. 1996; Hillebrand et al. 1996; Vauterin et al. 1999
		tCCAAc	−907	AT	Caubet et al. 1996; Hillebrand et al. 1996; Vauterin et al. 1999
		CAAT	−763	NT	Klotz and Lagrimini 1996
		CAAAt	−691	BR	no reference in database
		gCCAAt	−633	AT	Caubet et al. 1996; Hillebrand et al. 1996; Vauterin et al. 1999
		CCAAt	−632	BO	Pastuglia et al. 1997
		CAAT	−631	HV	Straub et al. 1994
		CAAT	−543	NT	Klotz and Lagrimini 1996
		gCCAAc	−495	AT	Caubet et al. 1996; Hillebrand et al. 1996; Vauterin et al. 1999
		CAAAt	−488	PS	Cocherel et al. 1996
		CAAT	−480	NT	Klotz and Lagrimini 1996
		aCCAAg	−448	AT	Caubet et al. 1996; Hillebrand et al. 1996; Vauterin et al. 1999
		TGCCgac	−441	PH	Solano et al. 1995
		gCCAAg	−427	AT	Caubet et al. 1996; Hillebrand et al. 1996; Vauterin et al. 1999
		cCCAAa	−334	AT	Caubet et al. 1996; Hillebrand et al. 1996; Vauterin et al. 1999
		CAAAt	−332	BR	no reference in database
		TGCCacc	−293	PH	Solano et al. 1995
		TGCCcac	−266, −257	PH	Solano et al. 1995
		gCCAAc	−205	AT	Caubet et al. 1996; Hillebrand et al. 1996; Vauterin et al. 1999
		TGCCaag	−188	PH	Solano et al. 1995
		gCCAAg	−187	AT	Caubet et al., 1996; Hillebrand et al. 1996; Vauterin et al. 1999
		CAAT	−149, −52	NT	Klotz and Lagrimini 1996
Core promoter element	TATA-box	acTATA	−1598	BN	Keddie et al. 1992
		cgTATA	−1098	BN	Keddie et al. 1992
		TATAca	−1096	HA	Coca et al. 1996; Carranco et al. 1997; Prieto-Dapena et al. 1999
		ccTATA	−983	BN	Keddie et al. 1992
		TATAttaa	−981	ZM	Arguello-Astorga and Herrera-Estrella 1996;
		TATA	−620	CR	Manjunath and Sachs 1997
		ccTATA	−87	BN	Pasquali et al. 1999
		cTATAat	−86	BO	Keddie et al. 1992
		TATAatt	−85	BJ	Dasgupta et al. 1993; Pastuglia et al. 1997
					Dasgupta et al. 1993
Transcription start site		CAC	−58		
conserved in alpha-amylase promoters	A-box	CCGTcc	−1559, −1127	PC	Logemann et al. 1995
		TAGCcaactag	−1021	OS	Kim et al. 1992
MeJA responsiveness	CGTCA-motif	CGTCa	−1578, −1526, −1254, −1172, −825, −766, −694, −55	HV	Rouster et al. 1997
Gibberellin responsiveness	P-box	CCTTtta	−751	OS	Kim et al. 1992; Washida et al. 1999
	TATC-box	TATCcca	−1504	OS	Washida et al. 1999

Table 2 (continued)

Function	PlantCARE	Sequence	Positions from ATG	Organism	Reference
		TATCcta	−986	OS	Washida et al. 1999
		TATCcct	−871	OS	Washida et al. 1999
Wound responsiveness	WUN-motif	tCATTccta	−823	BO	Pastuglia et al. 1997
		cCATTgcgt	−237	BO	Pastuglia et al. 1997
		tAATTtctc	−83	BO	Pastuglia et al. 1997
Dehydration, low temperature, salt stresses response	DRE	TACCgtcat	−828	AT	Yamaguchi-Shinozaki and Shinozaki 1994
Anoxic-specific inducibility	GC-motif	agCGCGtg	−1401	OS	Litts et al. 1992
		acCACGt	−1176	ZM	Arguello-Astorga and Herrera-Estrella 1996; Manjunath and Sachs 1997
		gcCACGa	−393	ZM	Arguello-Astorga and Herrera-Estrella 1996; Manjunath and Sachs 1997
Abscisic acid responsiveness	ABRE	accACGTcaa	−1176	Hv	Straub et al. 1994; Shen and Ho 1995
Endosperm-specific expression	GCN4_motif	ttaGTCA	−548	OS	Kim and Wu 1990; Washida et al. 1999
	Skn-1_motif	GTCAt	−824	OS	Washida et al. 1999
Heat stress responsiveness	HSE	aTAAAattt	−681	BO	Pastuglia et al. 1997
		aGAAAattt	−651	BO	Pastuglia et al. 1997
Light responsiveness	ACE	tcgACGTcgt	−1261	PC	Feldbrugge et al. 1994; Feldbrugge et al. 1996
	G-box	CACGtc CACGtc	−1174 −1174	PS ZM	An et al. 1993; Arguello-Astorga and Herrera-Estrella 1996; Ito et al. 1997; Bodeau and Walbot 1996; Arguello-Astorga and Herrera-Estrella 1996; Manjunath and Sachs 1997
		CACAtg CATGtc	−892 −1242	PS ZM	An et al. 1993; Arguello-Astorga and Herrera-Estrella 1996 1996; Ito et al. 1997; Bodeau and Walbot 1996; Arguello-Astorga and Herrera-Estrella 1996; Manjunath and Sachs 1997
		ccACGTca	−1175	BN	Keddie et al. 1992
		ctGGTG	−1163	HV	Arguello-Astorga and Herrera-Estrella 1996; Rouster et al. 1997
		CATGtg	−1027	ZM	Bodeau and Walbot 1996; Arguello-Astorga and Herrera-Estrella 1996; Manjunath and Sachs 1997
		CATGtc	−839	ZM	Bodeau and Walbot 1996; Arguello-Astorga and Herrera-Estrella 1996; Manjunath and Sachs 1997
		gaaACGTc	−698	BO	Pastuglia et al. 1997
		CACGac	−391	ZM	Bodeau and Walbot 1996; Arguello-Astorga and Herrera-Estrella 1996; Manjunath and Sachs 1997
		ccCGTG	−282	HV	Arguello-Astorga and Herrera-Estrella 1996; Rouster et al. 1997
	I-box	cATCAttt	−1483	ST	Arguello-Astorga and Herrera-Estrella 1996
		cATCAtgt	−1245	ST	Arguello-Astorga and Herrera-Estrella 1996
		gAGATaa	−1213	PS	Arguello-Astorga and Herrera-Estrella 1996
		AGATaaga	−1211	TA	Arguello-Astorga and Herrera-Estrella 1996
		GATAagaga	−1210	GM	Arguello-Astorga and Herrera-Estrella 1996
		tACATga	−1068	PS	Arguello-Astorga and Herrera-Estrella 1996
		GATAagaca	−1063	GM	Arguello-Astorga and Herrera-Estrella 1996
		tATATta	−981	PS	Arguello-Astorga and Herrera-Estrella 1996
		tATGAtat	−945	ST	Arguello-Astorga and Herrera-Estrella 1996
		gATATct	−942	FT	Arguello-Astorga and Herrera-Estrella 1996
		aATAAgtgt	−762	AT	Arguello-Astorga and Herrera-Estrella 1996
		gATAAttgt	−742	AT	Donald et al. 1990; Arguello-Astorga and Herrera-Estrella 1996
		gAAATaa	−602	PS	Arguello-Astorga and Herrera-Estrella 1996
		aACATga	−100	PS	Arguello-Astorga and Herrera-Estrella 1996

Table 2 (continued)

Function	PlantCARE	Sequence	Positions from ATG	Organism	Reference
		tATAAttt	−85	ST	Arguello-Astorga and Herrera-Estrella 1996
		tACATga	−29	PS	Arguello-Astorga and Herrera-Estrella 1996
	TCCC-motif	TCTCccc	−538	SO	Arguello-Astorga and Herrera-Estrella 1996
Unknown	Plant-AP2-like	cgtcCTGG	−1126	OS	Litts et al. 1992
	3-AF1-binding site	AAGAgagata AAGAgacagt	−1216 −1207	ST	not present in a gene
	BoxI	TTTCaag	−729	PS	Green et al. 1987
		TTTCaga	−614	PS	Green et al. 1987
	BoxII	CCACttacc	−1182	AT	Arguello-Astorga and Herrera-Estrella 1996
		ACACctata	−90	AT	Arguello-Astorga and Herrera-Estrella 1996
	C-repeat/DRE	atgCCGAc	−442	AT	Baker et al. 1994; Stockinger et al. 1997
	GC-repeat	cCGCCtag	−1466	OS	Litts et al. 1992
		gCGCCtcg	−1455	OS	Litts et al. 1992
		gCACCttg	−1394	OS	Litts et al. 1992
		aCGCCtag	−813	OS	Litts et al. 1992
		tCGCCacg	−395	OS	Litts et al. 1992
		tCGCCtcg	−344	OS	Litts et al. 1992
		gCGCCatg	−299	OS	Litts et al. 1992
		cCACCatg	−263	OS	Litts et al. 1992
		tCTCCagg	−114	OS	Litts et al. 1992
	TC-rich_repeats	aTTGTcttga	−668	NT	Klotz and Lagrimini 1996
		aTTCTctcat	−592	NT	Klotz and Lagrimini 1996

NT Nicotiana tabacum, AT Arabidopsis thaliana, BR Brassica rapa, BO Brassica oleracea, *HV Hordeum vulgare*, *PS Pisum sativum*, *PH Petunia hybrida*, *BN Brassica napus*, *HA Helianthus annuus*, *ZM Zea mays*, *CR Catharanthus roseus*, *BJ Brassica juncea*, *PC Petroselinum crispum*, *OS Oryza sativa*, *ST Solanum tuberosum*, *TA Triticum aestivum*, *GM Glycine max*, *FT Flaveria trinervia*, *SO Spinacia oleracea*, *GC-r* GC-repeat, *Wun-m* WUN-motif, *TC-rich-r* TC-rich-repeat, *CGTCA-m* CGTCA-motif, *TCCC-m* TCCC-motif, *GC-m* GC-motif (*cis*-acting elements in the 5′ region of 183-5; positions are numbered according to the position of the ATG)

transcription start site was detected at −58 (Table 2). A-box is a conserved element in alpha-amylase promoters. Three elements were detected at −1559, −1127, and −1021. Methyl jasmonate, MeJA, response element (CGTCA) was detected eight times at positions from −55 to −1578 (Rouster et al. 1997). P-box and TATC-box which respond to gibberellic acid are present at one and three times, respectively (Kim et al. 1992; Washida et al. 1999). Wound motif is repeated three times. DRE element was detected once at −828 (Yamaguchi-Shinozaki and Shinozaki 1994). GC-motif is present three times. ABRE is represented once and endosperm-specific expression elements GCN4 and Skn-1 are present at −548 and −824, respectively (Kim and Wu 1990; Washida et al. 1999). Four *cis*-acting elements involved in light responsiveness, ACE, G-box, I-box, and TCCC-motif, were also detected one, 11, 16, and one time, respectively. Some of the detected *cis*-acting elements are overlapped with similar elements from different promoters or with other elements. For example, three TATA-boxes are detected at −85, −86, and −87 from different Brassica species (Fig. 4)

The 717 bp coding for the ORF238 was used for a database search. It shows 52% similarity to EST sequence (AC# GI 21318924) derived from *Secale cereale* anther cDNA library and 44% to rice predicted protein (AC# OSJN00167) (Fig. 5)

Discussion

Sixty cDNA fragments were recovered from DD gels; 50% are from anther tissues. This may indicate that anther tissues are more distinct and complex in their development and that they require a higher number of novel gene expression than ovaries and leaves.

Reverse Northern blot revealed that AF18 and AF22 were among the fragments that showed differential response to total cDNA probes which make them good candidates for anther-specific gene expression, but this does not eliminate the possibility that other fragments are tissue specific. Many approaches have been developed to test tissue specificity of differential display fragments as early

Fig. 4 Demonstration of *cis*-acting elements detected in the 5′ region of 18-3 to show their location on the DNA sequence. *GC-r*, GC-repeat; *Wun-m*, WUN-motif; *TC-rich-r*, TC-rich-repeat; *CGTCA-m*, CGTCA-motif; *TCCC-m*, TCCC-motif; *GC-m*, GC-motif. Putative transcription start site is *double underlined*. When there is more than one *cis*-regulatory element in the same region, another row is drawn so that the line under the name exactly matches the corresponding sequence

```
-1674     TCCGCCAGTCCGTTGTCCCATGCTCTGTCGAAAGCCTGTAAGTTTTTTGTTGCTTGGACA
                         TATA               CGTCA-m             A-box
-1614     AAACTATCCATAGTTACTATACGGGGCATCCAGTCCGTCAGTTTTTTTGCACTACCGTCC
              CAAT                   CGTCA-m              TATC-box
-1554     AGTTCAATTTGGCACTTTTGTCCAGCCCGTCAGTTTTTTTGGACAAAACTATCCCATAGT
                    I-box            GC-r       GC-r
-1494     TACATGCGTCCATCATTTATGGATCGACCGCCTAGTAGGCGCCTCGAGAGTTACCTGACG
                                          GC-m   GC-r
-1434     GTGTCCATGAAGTACCGGTTCTCCGGCGACAGAGCGCGTGCACCTTGCTGGGGCTGCTAG
-1374     GGCCAGGTCTTCATGGACAGCGTCTCATTGGACGCCTGACATGCGCCTCTTCACTTTGGC
                                                              ACE
-1314     ATCCACAACAGCTTTGTGTTGCTGGTCTGCGAGCAACGCAACGAGGCATGCCTCGACGTC
                                                    I-box
                                                    I-box
                     G-box                           I-box
          CGTCA-m I-box  CAAT                  AF1-BS   AF1-BS
-1254     GTCAAGCTCATCATGTCCAAAGAGTGAGGGAAGTTCTAAGAGAGATAAGAGACAGTGTCC
                              G-box
                             G-box
                              ABRE
                            GC-m
                     BoxII     CGTCA-m  G-box
-1194     CGGCTAAGGGGCCACTTACCACGTCAACTCCTGGTGATGGGTCCGGTTGAGGACTCCTTG
                  Plant-AP2-like                TATA
                A-box                        TATA
-1134     TCAGTTCCGTCCTGGCCGCATCGGGCAGCCCATGGCGTATACAAGGAAGATTCAGAAGAC
                                                              A-box
               I-box I-box                              G-box   CAAT
-1074     ATGCGTACATGATAAGACACTGGATTCATGGAGGGATCTACCTCCTCATGTGTAGCCAAC
                                          I-box
          ___                            TATA
                                     TATC-box TATA
-1014     TAGGATCTTGTACCCTAGAACCCTTAGTATCCTATATTAATCGAGGGGCCATGCTCGTAG
                  I-box I-box                           CAAT
-954      ACACAAGTTATGATATCTTGGTAGTTTACCTGTACTCTGTCCATAGTCCAACAGGAATAA
           G-box                 TATC-box                       G-box
-894      ACACATGTAGGACTTAGGGTATTATCCCTTCAGAAAGCTTGAACTTGGGTAAACCATGTC
                  Skn-1-m
                    Wun-m
               DRE CGTCA-m    GC-r
-834      TTTGTTACCGTCATTCCTAGACGCCTAGTTTAGGATCCCCTACCAGGAGGTCTGCCAGAT
                 CGTCA-m
                    CAAT I-box  P-box    I-box        Box-I
-774      CTAGCTCCGTCAATAAGTGTTTCCTTTTAGGGATAATTGTATATTTTCAAGTAGCACTCA
                             CGTCA-m
                         G-box  CAAT      HSE          TC-rich-r
-714      ACAANGAAATGGATGGAAACGTCAAATAGGGCATAAAATTTTGAGATTGTCTTGAATAGC
                                CAAT
                               CAAT
            HSE               CAAT         TATA  Box-I       I-box
-654      AAAGAAAATTTTGTTTAGGGGCCAATAAGGAAATATATTTTTCAGAAGGGCGAAATAAGG
           TC-rich-r                                 GCN4-m CAAT  TCCC-m
-594      AATTCTCTCATCTCCTGCAGAGTTAGACCTTGCAAAAGAACCATCTTAGTCAATTTCTCC
                                                CAAT   CAAT    CAAT
-534      CCTTTCTATCGTCTCCTCCGCTCCCCTTCGCCGCCGCCGCCAACACAAATCTACAATTTT
                                         C-r-DRE
                                   CAAT   CAAT          CAAT
-474      ATCTTGTTCCTGGAAGGGAGCAACAACCAAGATGCCGACGACCATGGCCAAGGCCGCAAG
                              GC-m
                            GC-r G-box
-414      GGCAGGAGCGGCGCCTCTTCGCCACGACGGTAGCCTTCCGGAGGAGATCGTCGTCTGGGA
                               CAAT
                   GC-r      CAAT                               GC-r
-354      GATCCTAGTTCGCCTCGACCCCAAATCCCTCCTCCGTTGCCGCGTCGTCCGTCTGCGCCA
                                        GC-r
          CAAT       G-box           CAAT     CAAT                Wun-m
-294      TGCCACCTCCACCCGTGCCTTCCTGCTTGCCCACCATGCCCACCAGCCAGCCCTCCCCAT
                                                        CAAT
                                      CAAT             CAAT
-234      TGCGTTAGACGAAGAATACATCCTCACCGCCAACCGCCTTGCCGCTGCCAAGCTCCACAC
                                  CAAT
-174      TGTCGCCCATCTTGACAAGGCCTTCAATCCAGAGGCTTCCTGCGATGGCCTTCTCGTCCT
                                 BoxII
                                      TATA
                                     TATA
                                        I-Box
          GC-r         I-box          TATA  Wun-m                   CGTCA-m
-114      CTCCAGGTACAACAACATGACTGACACCTATAATTTCTCAGTTTGTAACCTGGCCACTCG
           CAAT                   I-box
-54       TCAATTTGCTCCCCTTCAACAACNTACATGAACTGGGCTTGCGGTTATTGGGGATGTACT
                                                               M  Y  S
          CGCATCAACCTACTGGCGAGTAT
```

Fig. 5 Amino acid alignment of ORF238 with EST database sequence from *Secale cereale* anther cDNA library (**a**) and a predicted protein from *Oryza sativa* (**b**)

a

```
ORF238: 235 LQSESKAIIVFDTIAESFRPMHAPIVSSNSYAFEMDGTLGIYSRNYAKTTIDIWVLQNYE 414
            L  E   I+VFDTI ESFR + AP+V   +  FEMDG LG+ S N A TTI+IWVLQNYE
SCEST : 632 LGRERSVIVVFDTIDESFRHIRAPVVPGRADVFEMDGMLGLSSFNDAATTINIWVLQNYE 453

ORF238: 415 SEVWDFKYRIKLPVAEIRGKFEAFDDHWNVEVVSADGDVLLLVNSGRCLSYVDNDGKLID 594
            SE W FK++I LP+ EIR K E  DD  NV VV  +  +L+LV     +  VD DGK+++
SCEST : 452 SEAWTFKFQIVLPLMEIRSKCENHDDRCNVVVVRGNDKLLVLVKFSEWMIEVDMDGKVVN 273

ORF238: 595 SF...RKYFFLSKYRLKQSLVQHTFFXXXXXXXXXXSPFI 714
            +F...RK    ++++LKQSLVQH FF           PFI
SCEST : 272 TF...RKDLRHTQFQLKQSLVQHAFFPRMRGYVVNRVPFI 159
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b

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ORF238: 1   MYSHQPTGEYRLLLERDSYNETPEDQL-------GYYVFALGSHQSPRYIGWQESTSQCF 159
            +Y H PTG+YR+LL R +     ED +         YV+ LGS+  PR IGW E
OSPP  : 154 LYQHSPTGDYRILLYR-AEKVVLEDLIPGHVERDASYVYTLGSNDMPRRIGWAEPEVSML 212

ORF238: 160 SLPVLVC-------DSLHWYPXXXXXXXXXXRLQSESKAIIVFDTIAESFRPMHAPIVSS 318
            +     C         LHWY              S    I+VFDT AESFR M API  +
OSPP  : 213 AGHSRRCRPAQLHGSLLHWY-------------HSIKHMILVFDTTAESFRWMRAPIDKT 259

ORF238: 319 NSY--------AFEMDGTLGIYSRNYAKTTIDIWVLQNYESEVWDFKYRIKLPVAEIRGK 474
             +           EMD TLG+Y  N+ KT ++IW LQ+YE EVW   KY+++LPV  IRG+
OSPP  : 260 ENELNRELWADVLEMDSTLGLYCCNHDKTIVNIWALQDYEQEVWSIKYQVELPVTCIRGE 319

ORF238: 475 FEAFDDHWNVEVVSADGD--VLLLVNSGRCLSYVDNDGKLIDSFDHGRKYFFLSKYRLKQ 648
             +   D W+V V S DGD  V++LV+ G+ +   D DGKL+   +H      +++ +LKQ
OSPP  : 320 LDV-GDSWSVMVSSEDGDEVVVVLVDCGQSVLCFDTDGKLLARLEHDGNDIMVTRMKLKQ 378

ORF238: 649 SLVQHTFFQALESSAVNTSPFI 714
            SLV H FF  L+S  VN  PFI
OSPP  : 379 SLVPHAFFPLLKSYVVNDLPFI 400
```

as possible to avoid using Northern blots with all fragments; this is impractical with a large number of samples and probably low expression. Reverse Northern blots (Zegzouti et al. 1997), Southern blots (Martin-Laurent et al. 1995), affinity capturing (Li et al. 1994), and a combination of differential display followed by differential screening (Zhang et al. 1996) have been used. All of these approaches have their own drawbacks and there is no thorough way to get rid of all false positives or confirm the real positives early. In reverse Northern blots, the use of colony lifts may lead to loading of unequal bacteria on the duplicate filters, causing false differential expression. Also, low expression of some genes makes them difficult to detect (Zegzouti et al. 1997). So, other factors should be considered when deciding between real and false positives of differential display fragments which include the appearance on DD gel, similar hits in the database, and the novelty of the sequence. Northern blotting showed that the message of AF18 and AF22, respectively, are expressed as RNA only in anther tissues (Fig. 3). This represents one more clear evidence that these two fragments are derived from anther-specific genes.

Database searches using AF18 and AF22 sequences showed that AF18 has 69% identity to *A. thaliana* sequence from spike before anathesis (AC # BQ170375) and 73% identity to rice pollen sequence (AC # BE225318), respectively. Therefore, based on their appearance on the DD gel, reverse Northern and Northern blot results, and database similarities, it seems that the promoter of the AF18 and AF22 fragments have a very high potential to be anther specific.

Genomic Library Screening

Sequence analysis of the obtained 3.7 kb from sequencing of clone 18-3 revealed that it has the general pattern of eukaryotic genes represented in promoter signals followed by an ORF of 238 aa. A putative transcription start site and TATA-box were detected by PlantCARE at their expected locations. Transcription start site (TSS) was detected at −58 from the ATG of the ORF238. The first TATA-box is located at −85 from the ATG of ORF238 and at −27 from the TSS, although there are multiple other TATA-boxes detected. The position of these two basic promoter elements strongly supports that there is a promoter structure upstream of ORF238. Many *cis*-acting elements in the 5′ flanking region involved in the plant response to a wide range of stimuli were determined by PlantCARE.

Having many different *cis*-acting elements in the 5′ flanking region isolated in this study indicates that it has complicated gene expression control over the ORF238. This also may give an idea that the function of the ORF238 could be essential for anther development or function in response to various stimuli. Complex promoters that respond to internal and external stimuli have been reported. Terpenoid synthases promoters showed temporal and spatial expression pattern (Cinege et al. 2009). Some of these gene promoters are induced by biotic and abiotic stresses (Yin et al. 1997; Fares et al. 2008). Isoperene synthase, the last enzyme for isoperene production, has a leaf-specific function. Its expression in White poplar (*Populus alba*) is induced by heat stress and light (Sasaki et al. 2005; Loivamaki et al. 2007).

Copy number, location, orientation, and proximity to other promoter components of *cis*-acting elements in a promoter have major a role in the regulation of transcription. Interaction of different *cis*-acting elements was studied in synthetic promoters (Cazzonelli and Velten 2008). Multiple G-box (CACGTG-motif, eight copies), in synthetic promoter enhanced the transient expression of reporter gene (Cazzonelli and Velten 2008). Higher-order modular structure of promoter elements has been reported in native promoters (Arguello-Astorga and Herrera-Estrella 1996) and synthetic promoters (Cazzonelli and Velten 2008). It is well established that light-induced promoters have complex structure which contain various *cis*-acting elements arranged in modular structures to coordinate gene expression in response to light. A large number, 110, of light-regulated plant genes were analyzed and 30 distinct conserved DNA module arrays (CMAs) associated with light-responsive promoters were identified. Some of these CMAs are conserved during evolution of angiosperms (Arguello-Astorga and Herrera-Estrella 1996).

The putative 5′ flanking region isolated in this study could be anther specific because (1) the cloned differential display fragment used for isolation of this region is differentially expressed on DD gels, (2) only clones of this fragment gave signal on reverse Northern blot, (3) on Northern blot, it is clear that the 18-1 fragment is driven from an anther-specific RNA message, (4) the isolated genomic DNA clone from wheat genomic library has the general eukaryotic gene organization of having ORF238 and upstream 5′ flanking region with promoter signals determined by PlantCARE, (5) of the presence of many different *cis*-acting promoter elements varying in number and organization in the 5′ region of ORF238, and (6) of the similarity of the 5′ flanking region to known complex promoters that respond to internal and external stimuli. Further analytical studies on this putative anther-specific sequence are needed to investigate (1) the functional/structure analysis of the putative promoter, (2) the possible function of ORF238, (3) if the different TATA-boxes in the sequence are functional and their comparative level of controlling gene expression, and (4) the promoter function in relation to environmental stimuli and anther as well as pollen development. Studying the characteristics of the promoter could reveal a multimodular promoter that could be related to the response of anther and/or pollen development to different stimuli as well.

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