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Full Length Research Paper

Extraction of differential expressing aphid-resistance genes of sorghum (*Sorghum bicolor* L. Monech) and construction of suppression subtractive hybridization (SSH) library

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Experiments were conducted for the extraction of differential expressing aphid-resistance genes of sorghum (*Sorghum bicolor* L. Monech) in the experimental laboratories and fields of Hebei Agricultural University, China and Shenyang Agricultural University, Liaoning Province, China, during 2010 to 2011 and suppression subtractive hybridization (SSH) library was constructed. The seeds of two sorghum varieties (Henong-16 and Qian-3) were grown and aphids were infested through natural and artificial way on sorghum seedlings (10-day old) with a paint brush. Total mRNA was isolated from fresh leaves samples using Trizol reagent and plant RNA mate (TAKARA). Integrity of RNA was confirmed by 1.2% agarose gel electrophoresis. SSH was performed using PCR-Select cDNA subtraction kit user manual according to the manufacturer's instruction (Clontech Laboratories, Inc, USA). cDNA that contained specific (differentially expressed) transcripts were denoted as tester and the reference cDNA as driver. Tester and driver cDNAs were hybridized after two rounds of subtractive suppression PCR and the pMD18-T vector (TAKARA, Dalian, China). After preliminary screening by subtractive hybridization, plasmid restriction enzyme digestion, colony PCR for 100 forward and 100 reverse clones were sequenced by two-way hybridization using Mega BACE1000 to obtain better quality of 200 expressed sequence tag (EST) sequences. Cross-match software and ClustalW2 were used to obtain vector sequence shielding and multiple comparisons. Using BLAST at NCBI database for homology comparisons, it was concluded that a number of EST sequences which had different degrees of homology with known proteins or genes and another six EST sequences did not have any significant homology in the database. These sequences might have representation for new and unknown genes, or higher variability of non-coding region cDNA sequences.

Key words: Extraction, sorghum, SSH, aphid-resistance genes.

INTRODUCTION

Aphids (Order: Homoptera) are major insect pests of the world's agriculture which damage crops by removing photo assimilates and vectoring numerous devastating plant viruses. Many pest aphid species, along with several hundred other insect pests, are resistant to insecticides (Smith and Boyko, 2007). Every year the yield potential of many crops is reduced because of these insects (Carena and Glogoza, 2004). Zia et al. (1999) reported that aphid's population was increased for the last few years on many crops including wheat, maize, sorghum and barely, and attained the status of pest.

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Majority of aphids suck sap of the leaves and young shoots causing distortion, stunting and sometime premature leaves fall (Akhtar and Khaliq, 2003). The excreta of aphid (honey dew) serves as a substrate for growth of sooty mould, which hinders the photosynthetic activity of plants (Pathan et al., 2005).

Sorghum (*Sorghum bicolor* L. Monech) is one of the five top cereal crops in the world, along with wheat, oats, corn and barley. It belongs to Poaceae (Gramineae) family. It originated in Africa and has been cultivated in Egypt in antiquity. So far, the largest producer of sorghum in the modern era is still Africa, although the crop has spread to Asia and the Americas as well. Sorghum is a potential crop for moderately saline areas (Almodares and Sharif, 2007). Sorghum provides food, feed, fiber, fuel and chemical feedstocks across a range of environments and production systems. Worldwide, sorghum is the 5th most important grain crop grown based on tonnage. It is second only to maize as US fuel ethanol crops and very closely related to sugarcane, the world's most important biofuel crop. Sorghum is a drought-resistant low input cereal grain grown throughout the world. In most of the countries, it is used primarily as animal feed, but in Africa and India, it is used as human feed, where it is a staple food for millions of people (Agrama and Tunistra, 2003). It is one of the important crops that can be utilized for the production of bioethanol and electricity.

Several crop plant resistance (R) genes and R gene homologues are associated with plant resistance to aphids. Single R genes inherited as a dominant trait control aphid resistance in forages, fruit and vegetables (Smith, 2005). Salzman et al. (2004) identified a LRR-containing glycoprotein sequence that is differentially expressed in leaves of sorghum infested by *Schizaphis graminum*. LRR-containing glycoproteins are extra-cellular, membrane-anchored compounds that in some cases recognize specific tomato leaf mold pathogen *Cladosporium fulvum* (*Cf*)-encoded avirulence gene products. Results of Rooney et al. (2005) indicated that *Cf-2* and its *Avr2* protein trigger a hypersensitive (resistance) response that also requires an extracellular tomato cysteine protease *Rcr3*. The binding of *Avr2* with and resulting *Rcr3* inhibition are proposed as the event that enables the *Cf-2* protein to activate a resistance response. A sequence similar to the *Xa1* gene encoding the protein that confers resistance to bacterial blight by recognizing a pathogen elicitor was also found by Park et al. (2005) to be up-regulated by *S. graminum* feeding on sorghum. Expression profiling of sorghum genes associated with treatments by methyl jasmonate (MeJA), salicylic acid (SA), and aminocyclopropane carboxylic acid demonstrated that both synergistic and antagonistic effects appeared in the expression of genes induced by SA or MeJA (Salzman et al., 2005). Enzymes secreted from aphid stylets inactivate functions of plant defense molecules by combining reducing compounds in aphid saliva to the defense molecules with support of oxidases, leading to depolymerization of the plant defense molecules (Miles, 1999). On four sorghum lines showing different resis-tance to aphids, fungal infection, and mechanical wounding, the expression patterns and active location of enzymatic activity of chitinase (CHI) and beta-1,3- glucanase (BGL) were investigated (Krishnaveni et al., 1999). Both susceptible and resistant lines showed intense induction of both genes, but duration and cellular location of each enzyme differed with the levels of resistance and types of stress employed.

Suppression subtractive hybridization (SSH) using isolated mRNA plays an important role in molecular investigations of interesting agronomic traits (Xu et al., 2006). SSH is the most famous subtraction method used for separating DNA molecules that distinguish closely related DNA samples (Lukyanov et al., 1994; Gurskaya et al., 1996; Diachenko et al., 1996). Two of the main SSH applications are cDNA subtraction and genomic DNA subtraction. In fact, SSH is one of the most powerful and popular methods for generating subtracted cDNA or genomic DNA libraries. The SSH method is based on a suppression PCR effect and combines normalization and subtraction in a single procedure. The normalization step equalizes the abundance of DNA fragments within the target population, while the subtraction step excludes sequences that are common to the populations being compared. This dramatically increases the probability of obtaining low-abundance differentially expressed cDNA or genomic DNA fragments, and simplifies analysis of the subtracted library (Rebrikov et al., 2004).

SSH has been widely used in the study of gene expression differentiation in animals and plants (Li et al., 2004; Bouton et al., 2005). Patterns of gene expression for different plants can be compared using subtractive library construction (Hedrick et al., 1984). Yang et al. (2010) studied molecular mechanism of three pistils mutation in wheat by two forward subtractive cDNA libraries from two pairs of near-isogenic wheat lines, three Chuanmai 28 pistils (CMTP) and three Chinese Spring pistils (CSTP) using SSH. A total of 68 clones in CMTP lines and 197 clones in CSTP lines were identified as potentially over-expressed clones. 32 out of 68 clones in CMTP lines belonged to unknown proteins, while the remaining 30 clones shared homology to diverse classes of genes involved in protein modulation and protein synthesis, signal transduction, and ion transporters. Approximately 67% of genes in CSTP lines were either unclassified or had no matches ("no hits") in the database and about 33% of identified genes encoded polypeptides with known functions. Sequence comparisons of cDNA clones between the two forward cDNA libraries revealed that four genes encoding thioredoxin H, ubiquitin protein ligases, MCM2 and ubiquinol-cytochrome C reductase complex 14 kDa proteins, were over-expressed in both libraries.

Xiao et al. (2009) compared gene expression pattern

during seed development between two *Brassica napus* mutants. Using immature seeds (27 days after pollination) differentially expressed cDNA clones were identified by SSH. A total of 480 cDNA clones corresponding to 88 genes were found up-regulated and 18 genes down-regulated in seeds with high oleic acid content. Most of the differentially expressed genes are related to metabolism and regulation. Differential gene expression in *Diuraphis noxia* biotype 1-resistant wheat plants containing the *Dnx* gene and *D. noxia* biotype 1 feeding on *Dnx* plants was also investigated using SSH (Boyko et al., 2006). The derived subtracted cDNA library include sequences similar to *Pto* and *Pti1*, genes involved in gene-for-gene recognition of and resistance to bacterial speck disease in tomato, *Lycopersicon esculentum* (L.). *Pto*- and *Pti1*-like sequences contain an activation domain with conserved amino acid residues crucial for *avr* protein recognition and binding by *Pto*, and avr-Pto phosphorylation of Pti1. Wheat defense signaling is represented by sequences putatively involved in producing sterols, jasmonates, Ca^{2+}, abscisic and gibberellic acids.

In this study, the expression profiles of sorghum genes were identified in response to sorghum aphids for a better understanding of the molecular defense mechanisms of sorghum against aphids by construction of SSH.

MATERIALS AND METHODS

Seeds of two sorghum varieties, Aphid-resistance variety "Henong-16" (denoted as A) and susceptible variety "Qian-3" (denoted as B), were obtained from the Laboratory of Sorghum, Hebei Agricultural University, China.

Plant growth and aphid culture

The seeds of the two sorghum varieties (Henong-16 and Qian-3) were sown in the experimental field of Hebei Agricultural University. Aphids were infested through natural and artificial way. For infestation, aphids were placed on sorghum seedlings (10-day old) with a paint brush. To maintain heavy infestation, approximately 20 aphids were placed on each seedling. Fresh leaves samples from two varieties (A and B) were collected for extraction of mRNA after aphid infestation of 12, 24, 48 and 72 h.

Total mRNA extraction, isolation and purification from sorghum leaves

Fresh leaves of two sorghum varieties (A and B) were collected and frozen immediately in liquid nitrogen and stored at -80°C prior to use. Total mRNA was isolated from fresh leaves samples using Trizol reagent and Plant RNA mate (TAKARA). Integrity of RNA was confirmed by 1.2% agarose gel electrophoresis. Purity and concentration of extracted RNA was verified by UV-spectrophotometer. Purified mRNA was extracted from samples A and B using Oligotex mRNA Kits (Qiagen).

SSH Library construction

SSH was constructed using PCR-Select cDNA subtraction kit user manual according to the manufacturer's instruction (Clontech Laboratories, Inc, USA). cDNA that contained specific (differentially expressed) transcripts were denoted as tester and the reference cDNA as driver. Tester and driver cDNAs were hybridized after two rounds of subtractive suppression PCR and the pMD18-T vector (TAKARA, Dalian, China) was used to build bi-directional suppression subtractive cDNA library. PCR condition was 94°C for 5 min; 34 cycles of (94°C for 45s, 46°C for 30 s and 72°C for 50 s); and 72°C for 10 min. PCR products were visualized on 1.5% agarose gel to inspect the quality and quantity of PCR products. Moreover, library recombination rate was calculated through the blue-white selection. Randomly picked white clones, plasmid DNA were extracted for PCR amplification and electrophoresis.

DNA sequencing and sequence analysis

After preliminary screening by subtractive hybridization, plasmid restriction enzyme digestion, colony PCR for 100 forward and 100 reverse clones were sequenced by two-way hybridization using Mega BACE1000 to obtain better quality of 200 EST sequences. Cross_match software and ClustalW2 were used to achieve vector sequence shielding and multiple comparisons. Repetitive sequences were removed and selected fragments with size of more than 100 bp and effective sequences were selected and located in NCBI nucleotide and protein sequence databases for derived comparison. The database search was performed on the basis of the cDNA sequences using BLAST. All cDNA sequences were submitted to NCBI using BLASTx (www.ncbi.nlm.nih.gov).

Northern-blot analysis

Total RNA was isolated from seedlings collected after four different time points of aphids' infestation (12, 24, 48 and 72 h.). Approximately 10 µg of total RNA per sample was fractionated in a 1% agarose gel containing 1.1 M formaldehyde, and then transferred to Hybond-N+ membrane (Amersham Biosciences, Piscataway, NJ) using the alkaline solution (3 M NaCl and 0.01 N NaOH) transfer method. Probes were labeled with 32P-dCTP (Perkin-Elmer) using PCR amplification of cDNA inserts from the pCR2.1 vector and hybridized to the membrane soaked with 2 ml of the UltraHyb buffer (Ambion) at 42°C overnight. Then, the hybridized blots were washed with 2X SSC/ 0.1% SDS at 65°C and 0.1X SSC/ 0.1% SDS at 60°C and exposed on Kodak BioMax MS film (Kodak, Rochester, NY) at -80°C overnight.

RESULTS AND DISCUSSION

SSH library construction

Trizol reagent and plant RNA mate (TAKARA) was used to extract total RNA of aphid-resistant and susceptible sorghum leaves with 1.2% agarose gel electrophoresis. The result showed 28S, 18S rRNA bands and 5S rRNA weak bands, indicating integrity of total RNA (Figure 1). Equal mixture of "Henong 16" total RNA which were infested at 12, 24, 48 and 72 h was taken as tester and equal mixture of susceptible "Qian3" total RNA infested at 12, 24, 48 and 72 h as driver. Oligotex mRNA Kits (Qiagen) were used to isolate the mRNA through reverse transcription. Double-stranded cDNA synthesis and SSH was constructed. Then, on the opposite, equal mixture of the susceptible "Qian3"total RNA and total RNA "Henong

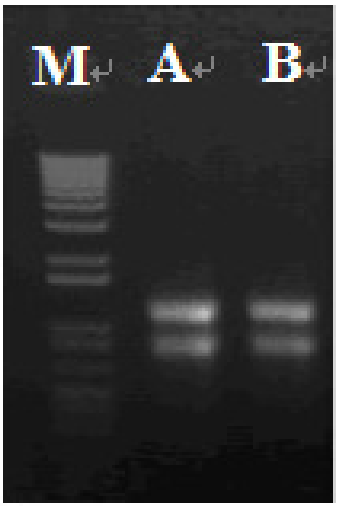

Figure 1. Total RNA electrophoresis. M, 10 Kbp DNA ladder; A, "HeNong16" total RNA; B, "Qian3" total RNA.

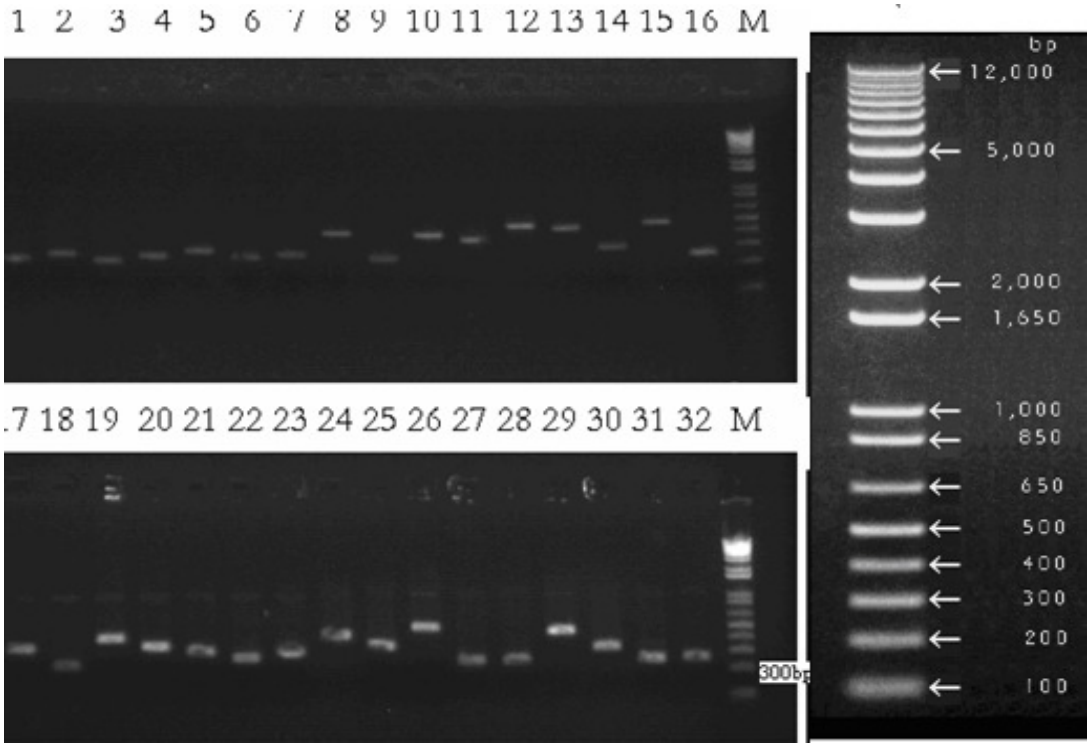

Figure 2. Detection of positive SSH library inserting size clones. 1 ~ 32, randomly picked library clones; M, 1 kb DNA ladder.

16" was used for reverse SSH; other steps were the same as used during positive SSH constructions. After two suppressions and two PCR amplifications, the size range of 300 bp of the amplified products was obtained (Figure 2), which had recombination rate greater than 95%.

Analysis of positive clones sequences

By subtractive hybridization and plasmid restriction enzyme digestion, colony PCR was sued for 100 forward clones and 100 reverse clones were one-way sequenced and 200 good qualities of EST sequences were obtained.

Table 1. Representative forward sequences/clones from SSH library matched through BLAST for aphid-resistance genes of aphid-resistance variety sorghum (Henong-16)

Clone number	Sequence detail	BLAST matching accession number	Gene description	E value	Maximum identification (%)
2	CTTTTGAGGGGATACATACCTTTCTTGCTCTTTTGAAACTTTTTGAGG AGAATGAGATGCTCTATATTTTCTTTTTTTTTCTCTCAGGCGGGTATCTC GTACCCCTAATTCTACTGTCGGACACTTGTCCATTTTTATCTCTCGTCT CACTTTTTTTTCTTTTCTTTCGAGGTTCCGGGCACTTGCCCCTTTTTA TTTCCTCATATTTTTTC	AC196837.2	Sorghum bicolor clone SB_BBc0073F19, complete sequence	3e-73	92
		AC196847.2	Sorghum bicolor clone SB_BBc0109L12, complete sequence	1e-76	92
3	ATCAGTTTTTGAGGTTGCCCAAAAAAAAAAGAAAAAGAAAGAAAAAAA AGAGAAAAGATTAAAAAAAAGGGGCTGTTTTTCATATTGATTTTAGGTT TGTTCCACCTTGTTTTTGGGGGTGTGCTGTGGTTTTCCTTTGTGTCC AGACTCGCATCTCTAGCACGGTCTAGCCTAGGACCAGCACAGT	AC169373.2	Sorghum bicolor clone SB_BBc0188M08, complete sequence	1e-67	92
9	ACTAGGTTGAATTACTATCGCGGCACGGTCATCAGTAGGGTAAAACTA ACCTGTCTCACGACGGTCTAAACCCAGCTCACGTTCCCTATTGGTGG GTGAACAATCCAACACTTGGTGAATTCTGCTTCACAATGATAGGAAGA GCCGACATCGAAGGATCAAAAAGCAACGTCGCTATGAACGCTTGGCT GCCACAAGCCAGTTATCCCTGTGGTAACTTTTCTGACACCTCTAGCTT CAAACA	XM_002488920.1	Sorghum bicolor hypothetical protein (SORBIDRAFT_1138s002030) mRNA, complete cds	2e-123	100
20	GACGATTAGCGTGGTCGCGGCCGAGGTTATTTTCACTCACTGTCCCG AATATTGTTATTCTCTCTCTAAAAAAAATCAATGCAGAAGAGGCATGG GTTATGCAAAAATATGCGAGGAAATAAAAAGGGGCAAGTTCCCGGAA CCTCGATAAAGAAAGAAAAAGAG	EU810765.1	Sorghum bicolor clone BAC Sbb12448, complete sequence	3e-48	92
		AY144442.1	Sorghum bicolor BAC 95A23/98N8.1 Rph region, partial sequence	1e-62	94
25	ACAAGCATTTTGTGTTTTTATTTTTTTTCTTATGCTTTTTACTCTAGACT TTTTTTATTTTTATCTATGTCATGTATTTATGTATTTTTATGTATATTGTAAT ACCAGTTTCATAAACCTAAAACCAAAATACTATTCTTCTAAATCGATAA CATTTTTTTACA	AY542311.1	Sorghum bicolor clone SB20O07 b1-1, b1-2, putative genetic modifier, hypothetical protein, putative NAM protein, putative cis-zeatin O-glucosyltransferases, putative small nuclear ribonucleoprotein, putative cis-zeatin O-glucosyltransferase, putative glutathione peroxidase, putative copper-exporting ATPases, putative serine/threonine dehydratase, and putative actin depolymerizing factor genes, complete cds; and hypothetical protein gene, partial cds	6e-65	95
		AY661659.1	Sorghum bicolor clone BAC 75D9, complete sequence	2e-55	93

Table 1. Contd

27	TACTTCCGGAGTAGAAGCAGCATGTGTGAGTGAACGTGCAAGTGAATCTTGATTTAACCACGTGACAAGCTCCTAAGGGTCTACACAGCTTGACCACACTCAATGCTCATAAGCAGTAAAAAGTAAATATGTGGCTCAAAGTCTAGCAAGCATGTATATTTGGCTGTGGTAGGAATTTAAACTCTCATCATACAGGAACTCATCGTGCAACATTTTAAAGATTTTCAGAAATAAAATTCTCCAGAATTCTAGCATCTCTAGGAACAGATAAACAGCAGCTCAACCTTCCCATATCATATCCGTTAACGACTTAGACTTTAGATCAAGT	AC169371.2	Sorghum bicolor clone SB_BBc0127F08, complete sequence	3e-142	94
29	GTTTTATATGCCTGATAAAACTGAAATATTAAATATGATTACAAAAGCTATCTGCTCTGTATTGAGTTTGTTCAAAATGAGTTAGACCTTTGTTGAGAGATTTATCATGCTCCTAAGATCAAGACATTTATTCAGAAAGATTCACTCTTCCGAAGTATTATTGCATTAGAGGCATGGGCTATGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGCTTGTA	AF466204.1	Sorghum bicolor clone SBTXS_0045I19, partial sequence	7e-79	95
36	ACAAGCTTTTTTTTTTTTTTTTTTTATTTATAATTTTGGTCAAACGTAAAAATTGTTTTGACTCTCCAAGATTCTTGGAATGACTTATAATTTGGGATGGATGAAGTATTTTAATAGTGGAATTATTGGGTGCTCCTCTCACCTCCTCTATTCTGATAGGTAGGCTGGGCCTAGGCAATGGTCTGTTGTGACATGAGCCTTCTTCCGACCCAACAAATGCAGAAAAGACCAAACAACACCGTCCAATGGCCTTTTTAGGATAGATTGGAAAAGAATATGGGCCGATATAGGAGGTCGGCAACACAACTGTTTGTTTGTTTTTTTTGTTTTTTTTTTTGGGGGGGGGGGGGGGGGGAGGAGG	AC169374.2	Sorghum bicolor clone SB_BBc0019I06, complete sequence	2e-19	90
42	GTATGAAGTATTAAATATAAATAAAAATAAAAACTAATTGCACAGTTTGGTCGGAATTGACGAGACGAATCTTTTGAGCCTAGTTAGTCCATGATTGGATAATTTTTATCAAATACAAACGAAAGTGCTACAGTGTCGATTTTGCAAAATTTTTTGGAACTAAACAAGGCCTTAATGTAAAACGTAGCAAAAAAAAGTCCCTCGC	AC169370.4	Sorghum bicolor clone SB_BBc0011I20, complete sequence	2e-60	91
		AC169372.2	Sorghum bicolor clone SB_BBc0156J18, complete sequence	1e-72	96
		AC169375.4	Sorghum bicolor clone SB_BBc0020O07, complete sequence	3e-62	97
		AC169377.4	Sorghum bicolor clone SB_BBc0068O12, complete sequence	1e-57	92
		AC169378.2	Sorghum bicolor clone SB_BBc0007L02, complete sequence	2e-65	93
		AC169379.4	Sorghum bicolor clone SB_BBc0088B22, complete sequence	1e-57	92
		AC188038.1	Genomic sequence for Sorghum bicolor BAC clone SB_IBa82G24, complete sequence	1e-71	95
		AF124045.1	Sorghum bicolor BAC clone 110K5, partial sequence	2e-60	92

Table 1. Contd

	AF466199.1	Sorghum bicolor putative receptor protein kinase, aminoalcoholphosphotransferase, putative growth-regulating factor 1, putative GAG-POL precursor, putative GAG-POL precursor, putative RIRE2 orf3, putative anthocyanin regulatory C1, putative protein T30F21.6, putative copia polyprotein, putative copia polyprotein, putative protein NP_196765.1, and gb protein genes, complete cds; and putative zinc finger protein gene, partial cds	4e-47	97
	AF466200.2	Sorghum bicolor putative protein kinase gene, partial cds; putative Cf-2, fertilization-independent endosperm proteins, hypothetical protein, putative non-LTR retroelement reverse transcriptase, OCL5 protein, tryptophan synthase beta-subunit, hypothetical proteins, putative AP endonuclease, putative RNA polymerase II complex component SRB7, putative beta-1,3-glucanase, hypothetical protein, TNP2-like protein, hypothetical protein, putative phosphate/phosphoenolpyruvate translocator, putative protein, hypothetical proteins, putative galactosyltransferase family, hypothetical protein, putative cytochrome P450 family, putative lipid transfer protein, putative photoreceptor-interacting protein, and hypothetical protein genes, complete cds; and hypothetical protein gene, partial cds		
	AF466201.1	Sorghum bicolor clone SBTXS_0032H17 putative cytochrome P450-like protein, putative DNA-binding protein homolog, TATA-binding protein, hypothetical protein M3E9.200, 3-glucanase, K-exchanger-like protein, small heat shock-like protein, methionine synthase protein, putative far-red impaired response protein, and putative vegetative storage protein genes, complete cds	3e-68	100
	AF488412.1	Sorghum bicolor BAC 131L1, complete sequence	7e-64	94
	AF527807.1	Sorghum bicolor clone BAC SB_BBc0126P21 php200725 orthologous region	6e-65	93
	AF527809.1	Sorghum bicolor clone BAC SB_BBc0234M12 php200725 orthologous region	6e-65	93

Table 1. Contd.

		AY661657.1	Sorghum bicolor clone BAC 60H10, complete sequence	4e-42	91
		AY661658.1	Sorghum bicolor clone BAC 796all, complete sequence	2e-54	91
		AY761821.1	Sorghum bicolor clone 152702 unknown sequence	3e-57	91
		AY761823.1	Sorghum bicolor clone 221540 unknown sequence	3e-57	91
		AY761824.1	Sorghum bicolor clone 221607 unknown sequence	3e-57	91
		AY761832.1	Sorghum bicolor clone 50875 unknown sequence	3e-57	91
		AY761841.1	Sorghum bicolor clone 83707 unknown sequence	3e-57	91
		BK007081.1	TPA: TPA_inf: Sorghum bicolor Dof-type zinc finger protein 27 (Dof27) gene, complete cds	3e-63	92
		EU583216.1	Sorghum bicolor cultivar Colby Pc gene cluster, complete sequence	1e-62	95
		FN431662.1	Sorghum bicolor BAC contig 24P17c, cultivar Btx623	3e-63	93
		XM_002457171.1	Sorghum bicolor hypothetical protein, mRNA	3e-43	92
50	TCAAGCTTTTTTTTTTTTTTTTTTTTTTTGTTTTTTTGATTTTTTTTTGTAAGC AAAGGAAACATAATACCTAAATTCTAAACAAAAGTAGAAGTTCAAAACCT AATAAATTTAATAATCTCTTCCATTCTATGGCCCCAAGAATGTCAACCT	EF115542.1	Sorghum bicolor cultivar BTx623 chloroplast, complete genome	1e-36	93
58	TGCATCTTTGTACCCCTAATTCTATTGTCGGACACTTGTCCATTTTTACC TCTCGTCTCACTTTTTCTCTTTTTCTTTCGAGGTTCCAGGCACTTGCCC CTTTTTATTTCCTCGTATTTTTCTTCTTTTTTTCAGAGCACTCACCCTCCT GGAATAATGTAGCAAGTGGTAGTAACCAAAATAACTTGAGCATTTATTTC CCAGGGAAAAATAGGAATGGGATAATGTTTTTGGCTATTCTCTCCCGGA TAGGAGTAGAATAATTTTGGGTGAATCTGGAGATGGAAATTTGTGGATG TATGTGGATGCATACTTCCGGAGTAGAAGCAACATGTATGAGTGGGCGT GCAAGTGAATCTTGATTATAACCGCATGACAAGCTCCTAAGGGTCTACA TAGCTTGACCACACTCAATGATCATAAGCAGTAAAAAGTC	AF061282.1	Sorghum bicolor 22 kDa kafirin cluster	6e-35	95

Table 1. Contd

64	TCTCTAAACCTAAAACTTGTGGGAAGAAAACTTGATCTAAAGTCTAAG TTGTTAACGGATACGATATGGGAAGGTTGAGCTGCTGTTTATCTATTC CTAGAGATGCTAGAATTCTGGAGAATTTTATCTTTCAAAATCTTTAAAAT ATTGCATGATGAGTTCCTATATGATGAGAGCTAGAATTCCTACCACAG CCAGATATACATGCCTTGTAGATTAGGAAAAACATTTACTTTTTACTGC TTATGAGCATTGAGTGTGGTCAAGCTGTGTAGACCCTTAGGAGCTTAT CATGTGGTTAAGATCAAGATTCACTTGCACGTTCACACACACATGCTG CTTCTACTCTGGAAGTACGCATCCACATATATCCACTCATTTCCATCTC CAGATTCACCCAAAATTATTCTACTCCTAATCTGGGAGAGAATAGC	AC196852.2	Sorghum bicolor clone SB_BBc0169M22, complete sequence	8e-168	91
84	GTTGGATGCCCGGGCATTGAGAAGGAAGGACGCTTTCAGAGGCGAA AGGCCATGGGGAGAGAGCGTCTGTGATCCATGGATCTCCGATCGGG AAACCGTATCCAAGCTCCGTGGCTAGTCTGCGCTCTTTGGACTTTTC AAACTTAGCGAACTGAAACATCTGAGTAGCTAAAGGAAGGAAAATCA ACCGAGACCCCGTTAGTAGCGGCGAGCGAGAGCGGAACAAGGGTT TTCATCAAAAGAAATCCGAAGCGGTTTCATTCGATTTGTTGTGGATTG GATGATGGAAAAACCAGCAAGCAGCAAGCGTAGGCTGTGTTGCCGT AGCGCGCCCTACGGAGTTGTA	DQ984518.1	Sorghum bicolor mitochondrion, complete genome	2e-179	100
87	GTGCTAGGCTAGCTGCAGCTCAGGTGCTTGTCTGAGGGTGCTGAGG GTGCCTCCTCATTCGCAACCGGATGATCGCCTGCTGTTGGCGCATCT GGTGATTAATAATATTGTTACAGAGCCATGATCTGTGAAGATAATTAGT AGCAGGGCTCATAAAAGCTACAATTCCATCCCTTTTTGCAGTTATGTA AAACTTTCAAACTGTTTATGCTCAAAAACTCTGTTCTTCAATGGATCAT CAATTATCGACCAAAAAAAAAAAAAAAAAAAAAAAAAAAAGCTTGTAC	AY268138.1	Sorghum bicolor pyruvate phosphate dikinase mRNA, complete cds	9e-128	98
88	GGTACCCCTAATTCTACTGCCGGACACTTGTCCATTTTTCTGCGAGG TTGTCGAGCACTTGCTCCTTTTTATTTCCTCGAAATATTTCTATTTTTG CATAGCCCATGCCCCTAATGCAATAATACTTCGGAAGAGTGAATCTTA CTGAAATAATGTCTTGATCTTGGGAGCATGATAAATCTCTCAACA	AC196829.2	Sorghum bicolor clone SB_BBc0050H06, complete sequence	3e-67	91
93	TACGTCGTGACTTTAAAGTCACCCAATCTCTGTCGGTAAGGAACGTG GCGCGTGGGCTTTTTATTTTTTATTTTATTTTTTTATAAGGCCTTGTTTA GATTGGAGATGAAAATTTTTTGGGTGTCACATCGGATGCGTCGGAAG GATGTCGAGAGGGATTTTTAGAAACTAATGAAAAAAAAAAAAAAAAAA AGCTTGTAC	AC169376.2	Sorghum bicolor clone SB_BBc0046M17, complete sequence	1e-27	91
99	TACTAGGTTGAATTACTATCGCGGCACGGTCATCAGTAGGGTAAAACT AACCTGTCTCACGACGGTCTAAACCCAGCTCACGTTCCCTATTGGTG GGTGAACAATCCAACACTTGGTGAATTCTGCTTCACAATGATAGGAA GAGCCGACATCGAAGGATCAAAAAGCAACGTCGCTATGAACGCTTG GCTGCCACAAGCCAGTTATCCCTGTGGTAACTTTTCTGACACCTCTA GCTTCAAACTCCGAAGGTCTAAAGGATCGATAGGCCACGCTTTCACG GTTCGTATTCGTA	XM_002488920.1	Sorghum bicolor hypothetical protein (SORBIDRAFT_1138s002030) mRNA, complete cds	3e-152	100

Table 2. Representative reverse sequences/clones from SSH library matched through BLAST for aphid-resistance genes of aphid- susceptible variety sorghum (Qian-3).

Clone number	Sequence detail	BLAST matching accession number	Gene description	E value	Maximum Identification (%)
3	TTTCGAGCGGCCGCCCGGGCAGGTGTGCTTTTTTTAAA AAAATGAGAGAAAAAGAA	AC196818.2	Sorghum bicolor clone SB_BBc0005H14, complete sequence	1e-41	95
		AC196837.2	Sorghum bicolor clone SB_BBc0073F19, complete sequence	2e-48	100
4	AAAAAAATATACGAGGAAATAAAAAGGAGCAAGTGCTCG GAACCTCGAAAGAAAAGAAAAAGTGAGACGAGAGGTAA AAATGGACAAGTGTCCGACAGTAGAATTAGGGGTACCT CGGCCGCGACCACGCTAA	AC196852.2	Sorghum bicolor clone SB_BBc0169M22, complete sequence	5e-45	95
		AF061282.1	Sorghum bicolor 22 kDa kafirin cluster	4e-41	94
		EU810765.1	Sorghum bicolor clone BAC Sbb12448, complete sequence	1e-30	95
7	TTTCGAGCGGCCGCCCGGGCAGGTCAGCAAGTCGCTC ACACTCAACCTGTAACACAAGTTCTTACCAATTCTTACC TTGCTTGACAGGAGGGGTCGTCTGCCAACAAGTGTAC CTCGGCCGCGACCACGCTAA	AC169373.2	Sorghum bicolor clone SB_BBc0188M08, complete sequence	9e-38	100
8	TACTTCCGGAGTAGAAGCAGCATGTGTGAGTGAACGTG CAAGTGAATCTTGATTTAACCACATGACAAGCTCCTAAG GGTCTACACAGCTTGACCACACTCAATGCTCATAAGCA GTAAAAAGTAAATATGTGGCTCAAAGTCTAGCAAGCATG TATATTTGGCTGTGGTAGGAATTTAAACTCTCATCATACA GGAACTCATCGTGCAACATTTTAAAGATTTTCAGAAATAA AATTCTCCAGAATTCTAGCATCTCTAGGAACAGATAAAC AGCAGCTCAACCTTCCCATATCATATCCGTTAACGACTT AGACTTTAGATCAAGTA	AC169371.2	Sorghum bicolor clone SB_BBc0127F08, complete sequence	6e-144	95
10	TACCATCCACATATATCCATCCATTTCCATCTCCAGAACC ACCCAAAAATATTCTACTCCTAATCCGGGAGAGAATAAC CAAAAATATTTCTGTTTTCCCCTTGTGAAATAAATGCTCA AGTTATCTTGTTACTACCACTTGCTATATTATCTCAAGAG ATGAGTGCTCTAAAAAAAATGAGGAAATAAAAAGGAGCA AGTGCTCGGAACCCCGAAAGAAAAGAAAAAGTGAGAC GGGAGGTAAAAATTGGAC	AC196847.2	Sorghum bicolor clone SB_BBc0109L12, complete sequence	6e-99	92
18	GTACTAGGCTGAATTACTATCGCGGCACGGTCATCAGTA GGGTAAAACTAACCTGTCTCACGACGGTCTAACCCCAG CTCACGTTCCCTATTGGTGGGTGAACAATCCAACACTT GGTGAATTCTGCTTCACAATGATAGGAAGAGCCGACAT CGAAGGA	XM-002488920.1	Sorghum bicolor hypothetical protein (SORBIDRAFT_1138s002030) mRNA, complete cds	7e-74	93

Table 2. Contd

		002447669.1	Sorghum bicolor hypothetical protein, mRNA	9e-23	100
		002447669.1	Sorghum bicolor hypothetical protein, mRNA	9e-23	100
		AC169369.2	Sorghum bicolor clone SB_BBc0115C15, complete sequence	2e-04	100
		AC169378.2	Sorghum bicolor clone SB_BBc0007L02, complete sequence	9e-18	92
		AF466199.1	Sorghum bicolor putative receptor protein kinase, aminoalcoholphosphotransferase, putative growth-regulating factor 1, putative GAG-POL precursor, putative GAG-POL precursor, putative RIRE2 orf3, putative anthocyanin regulatory C1, putative protein T30F21.6, putative copia polyprotein, putative copia polyprotein, putative protein NP_196765.1, and gb protein genes, complete cds; and putative zinc finger protein gene, partial cds	2e-04	100
32	TATTACCACAAACAAACGAAAGTGCTACAGTGTCACGAA ACTTTTTCATTCAGGAACTAAACAAGGCCTTGGTTGGTC AAAAATTCTGAAAAGCAGAATCTGACCACACACAAACA CACACACACACACAAAAAAAAAAAAAAAC	AF466200.2	Sorghum bicolor putative protein kinase gene, partial cds; putative Cf-2, fertilization-independent endosperm proteins, hypothetical protein, putative non-LTR retroelement reverse transcriptase, OCL5 protein, tryptophan synthase beta-subunit, hypothetical proteins, putative AP endonuclease, putative RNA polymerase II complex component SRB7, putative beta-1,3-glucanase, hypothetical protein, TNP2-like protein, hypothetical protein, putative, phosphate/phosphoenolpyruvate translocator, putative protein, hypothetical proteins, putative galactosyltransferase family, hypothetical protein, putative cytochrome P450 family, putative lipid transfer protein, putative photoreceptor-interacting protein, and hypothetical protein genes, complete cds; and hypothetical protein gene, partial cds	2e-19	94
		AY661656.1	Sorghum bicolor clone BAC 88M4, complete sequence	2e-14	100
		DQ459071.1	Sorghum bicolor cultivar BTx623 clone BAC c0156b06, complete sequence	2e-04	100
		XM-002436419.1	Sorghum bicolor hypothetical protein, mRNA	2e-04	100
58	TCTCGATCGGAAAAGAATCAATAGAAGGAGAATCGGAC GATATCTTTTTCGAAACAAATAAAAAGGAAAAAAAAAAG AGAAAACAGAAATCATGATCAACTAAGCCCTCTCGGGG GCTTGCTTAAGA	EF115542.1	Sorghum bicolor cultivar BTx623 chloroplast, complete genome	3e-57	99

Table 2. Contd

62	TACCCCTAATTCTACTGCCGGACACTTGTCCATTTTTTCTGCGAGGTTGTCGAGCACTTGCTCCTTTTTATTTCCTCGAAATATCTCTATTTTTGCATAGCCCATGCC	AC196829.2	Sorghum bicolor clone SB_BBc0050H06, complete sequence	2e-33	91
		AF466204.1	Sorghum bicolor clone SBTXS_0045I19, partial sequence	2e-48	100
81	TGAAGTATTTTTTCACTATACAAGACCCAAAATAGGTTTGTGTAACAGTTTGCATAGCTTGTTGGGGTTGGTCTAATAGAGCCAAAATGCGGCCTTGTTTATTTACATCCGAAATCCAAAAACTTTTCAAGATTCTCTATCACATTGAATCTTACGACACATGCATAAAGCATTAAATATAGATAAAAAATAACTAAT	AC169375.4	Sorghum bicolor clone SB_BBc0020O07, complete sequence	2e-20	91
83	TATTCTAGAGCTAATACGTGCAACAAACCCCGACTTCCGGGAGGGGCGCATTTATTAGATAAAAGGCTGACGCGGGCTCTGCTCGCTGATCCGATGATTCATGATAACTTGACGGATCGCACGGCCCTCGTGCCGGCGACACATCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGGGGCCTACCATGGTGGTGACGGGTGACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACGGGGAGGTAGTGACAATAAATAACAATACCGGGCGCGTTAGTGTCTGGTAATTGGAATGAGTA	XM-002488909.1	Sorghum bicolor hypothetical protein (SORBIDRAFT_1236s002010) mRNA, complete cds	0.0	100
		XM-002488912.1	Sorghum bicolor hypothetical protein (SORBIDRAFT_1205s002020) mRNA, complete cds	0.0	100
		XM-002488924.1	Sorghum bicolor hypothetical protein (SORBIDRAFT_1050s002020) mRNA, complete cds	0.0	100
		XM-002488980.1	Sorghum bicolor hypothetical protein (SORBIDRAFT_0450s002020) mRNA, complete cds	0.0	100
106	TCGAAGAAAAAGGTAAAAGGGTTTCAAATTAATAAAATAGAAGGAGGTTATTCAGTAGCCATCGCAGGTTTCATTACTTTTCTTCCATTCAAATTAAAAAAAGCTAAAAAAAAAAAAAAGGCTTGTAC	DQ984518.1	Sorghum bicolor mitochondrion, complete genome	2e-54	100
107	ACATCAAACGAGGAACTCTGCCACGTCATCCAGCGACTTCAACGTCTTCTCGTAGAAGAGGTAGGACTCGTAGACCTTGGGAGTCCGCACGAAGCGATCGAACTCCGTCATGTTGCCGACGAGCTCGTTAGCGACACGGACGTAGTCACCTGCCCGGGCGGCCGCTCGAAATCGTCGACCTGCAGGCATGC	XM-002454016.1	Sorghum bicolor hypothetical protein, mRNA	3e-67	99
		AY144442.1	Sorghum bicolor BAC 95A23/98N8.1 Rph region, partial sequence	9e-63	94
109	ACAAGCATTTTGTGTTTTTATTTTTTTTCTTATGCTTTTTACTCTAGACTTTTTTTATTTTTATCTATGTCATGTATTTATGTATTTTTATGTATATTGTAATACCAGTTTCATAAACCTAAAACCAAAATACTATTCTTCTAAATCGATAACATTTTTTTACA	AY542311.1	Sorghum bicolor clone SB20O07 b1-1, b1-2, putative genetic modifier, hypothetical protein, putative NAM protein, putative cis-zeatin O-glucosyltransferases, putative small nuclear ribonucleoprotein, putative cis-zeatin O-glucosyltransferase, putative glutathione peroxidase, putative copper-exporting ATPases, putative serine/threonine dehydratase, and putative actin depolymerizing factor genes, complete cds; and hypothetical protein gene, partial cds	5e-65	95

Table 2. Contd

		AY661659.1	Sorghum bicolor clone BAC 75D9, complete sequence	1e-55	93
112	TAAAAGTTTCAAAGAGCAAGAAAGGTATGTATCCCCTCA AAAGAGCAAAAGTAGAATTAGACTCTCACCATTGTTATC ACTATCATCACCATACACCATCCATTCGCCACACATGCA CATCTTGATTTGGCTTATTGATTTGTTTCTTTGGATCCAT GGTTTGACTATGCAATAAATGTCTTGTAAGTATGTGTAC	AC196818.2	Sorghum bicolor clone SB_BBc0005H14, complete sequence	1e-76	94

Cross-match software and ClustalW2 were used to get vector sequence shielding and multiple comparisons. After repetitive sequence remover and smaller similarity selection, 103 effective sequences with fragment size of more than 100 bp were obtained. Using BLASTx at NCBI database for homology comparisons, it was found that a number of EST sequences had different degrees of homology with known proteins or genes (Table 1), and another six EST sequences did not have any significant homology in the database; these sequences might have representation for new and unknown genes, or higher variability of non-coding region cDNA sequences. Results regarding forward and reverse sequences matching through BLAST at NCBI database for homology comparisons are given in Tables 1 and 2, respectively. It was noted that 17 forward sequences showed match for different gene descriptions from NCBI database expressing genes for sorghum and 15 genes of reverse (tester variety) matched with NCBI database.

Yang et al. (2010) studied molecular mechanism of three pistils mutation in wheat by two forward subtractive cDNA libraries from two pairs of near-isogenic wheat lines, three CMTP and three CSTP using SSH. A total of 68 clones in CMTP lines and 197 clones in CSTP lines were identified as potentially over-expressed clones. 32 out of 68 clones in CMTP lines belonged to unknown proteins, while the remaining 30 clones shared homology to diverse classes of genes involved in protein modulation and protein synthesis, signal transduction and ion transporters. Approximately 67% of genes in CSTP lines were either un-classified or had no matches (“no hits”) in the database and about 33% of identified genes encoded polypeptides with known functions. Sequence comparisons of cDNA clones between the two forward cDNA libraries revealed that four genes encoding thioredoxin H, ubiquitin protein ligases, MCM2, and ubiquinol-cytochrome C reductase complex 14 kDa proteins, were over-expressed in both libraries. Xiao et al (2009) also studied gene expression pattern during seed development between two *Brassica napus* mutants using immature seeds 27 days after pollination, and differentially expressed cDNA clones were identified by subtractive suppression hybridization (SSH). A total of 480 cDNA clones corresponding to 88 genes were found up-regulated and 18 genes down-regulated in seeds with high oleic acid content. Most of the differentially expressed genes were related to metabolism and regulation.

More also, two subtracted cDNA libraries of *Dunaliella salina* (Volvocales, Chlorophyceae) under different hyperosmotic shock were also constructed using the SSH method. The differentially expressed cDNA fragments in *D. salina* under salt stress were identified by screening these two libraries. Two cDNA fragments, *D27* and *D114*, were identified from clones pL27 and pL114 after the long-term treatment. Three cDNA fragments, *D21*, *D39* and *D88*, were identified from clones pSh21, pSh39, and pSh88 after the short-term treatment. The homology analysis revealed that D27 was highly similar (91%) to the subunit V of PS-I reaction center in *Chlamydomonas reinhardtii*. D21 was similar to 78.4% fructose-1,6-diphosphate aldolase (Zhang et al., 2002).

Conclusion

Using BLAST at NCBI database for homology comparisons, it was concluded that a number of EST sequences which had different degrees of homology with known proteins or genes and another six EST sequences did not have any significant homology in the database; these sequences might have representation for a new and unknown genes, or higher variability of non-

coding region cDNA sequences.

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