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Sequence similarity based identification of abiotic stress responsive genes in chickpea

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Abstract:
Chickpea (*Cicer arietinum L.*) is an important food legume crop, particularly for the arid regions including Indian subcontinent. Considering the detrimental effect of drought, temperature and salt stress on crop yield, efforts have been initiated in the direction of developing improved varieties and designing alternate strategies to sustain chickpea production in adverse environmental conditions. Identification of genes that confer abiotic stress tolerance in plants remains a challenge in contemporary plant breeding. The present study focused on the identification of abiotic stress responsive genes in chickpea based on sequence similarity approach exploiting known abiotic stress responsive genes from model crops or other plant species. Ten abiotic stress responsive genes identified in other plants were partially amplified from eight chickpea genotypes and their presence in chickpea was confirmed after sequencing the PCR products. These genes have been functionally validated and reported to play significant role in stress response in model plants like *Arabidopsis*, rice and other legume crops. Chickpea EST sequences available at NCBI EST database were used for the identification of abiotic stress responsive genes. A total of 8,536 unique coding long sequences were used for identification of chickpea homologues of these abiotic stress responsive genes by sequence similarity search (BLASTN and BLASTX). These genes can be further explored towards achieving the goal of developing superior chickpea varieties providing improved yields under stress conditions using modern molecular breeding approaches.

Keywords: Chickpea, Abiotic stress, Candidate genes, Sequence similarity

Background:
Chickpea (*Cicer arietinum L.*), a member of the *Fabaceae (Leguminosae)*, is the third most important food legume world-wide after pea and common bean, with over 10 million hectares under cultivation (FAO 2009). It is a self-pollinated, diploid (2*n* = 16) annual crop having genome size of ~ 740 Mbp. Chickpea has one of the most balanced nutritional compositions, and its protein digestibility is the best among the dry season food legumes. Chickpea seeds contain 20-30% protein, approximately 40% carbohydrates and many other useful nutrients. Chickpea fits well in crop rotation programs because of the ability of fixing atmospheric nitrogen. It leaves large amount of residual nitrogen behind for subsequent crops and adds much needed organic matter to maintain and improve soil health, long term fertility and sustainability of the ecosystem.
Chickpea is primarily a crop of developing countries contributing to a large part of human food and animal feed in these areas. Current global yield average of chickpea is 0.9 t/ha, is much lower than its estimated potential of 6 t/ha under optimum growing conditions (FAO 2009). India is the largest producer of chickpea, but still is the largest importer. Chickpea yield in India has remained at 0.89 t/ha, which is much lower than the maximum yield reported in china i.e. 3.2 t/ha (FAO 2009). Chickpea yield and productivity are adversely affected by various biotic and abiotic stresses like *Ascochyta* blight, *Fusarium* wilt, *Helicoverpa* pod borer, *Botrytis* grey mold, drought and cold **[1].** Abiotic stresses stand to be the number

one problem in chickpea growing regions causing a 40–50% reduction in yield globally **[2].** Considering the effect of drought, temperature and salt stress on yield, it is very important to intensify work in the direction of developing improved varieties or devising alternate strategies to sustain chickpea cultivation in adverse environmental conditions thereby improving chickpea production.

Application of available approaches to improve crop productivity under adverse environmental conditions requires a better understanding of the mechanisms involved during crop's response to abiotic stresses. Since response to abiotic stress is a complex phenomenon, information about gene(s) involved in the process is essential. Tracking the candidate genes responsible for stress tolerance through sequence similarity and functional studies is becoming increasingly important for marker-assisted breeding **[3].** Such candidate genes serve as useful resource for comparative genomics and can be further used as molecular markers or for genetic transformation to develop desired cultivars.

Conventionally, plant breeders aiming to introduce drought tolerance in crop plants used to exploit selection of yield and yield components under water limiting conditions. Such conventional efforts directed towards breeding drought tolerance did not meet desired success due to limited knowledge about the genetic basis of drought tolerance and its negative correlations with productivity. Recent advancement in the technology has allowed development of various genetic tools and genomic approaches to identify genomic regions and genes/ quantitative trait loci (QTLs) underlying plant stress response in many crop species **[4].**

Recent advancements in the sequencing technology has provided a rapid and cost effective method for generation of enormous sequence data facilitating identification of plant genes mediating stress tolerance. Researchers have concentrated on the identification of candidate genes involved in abiotic stress response in plants, which can further be used in crop improvement programs, directly through transgenic approach or indirectly exploiting SNPs associated with the desired trait. A number of abiotic stress responsive genes have been identified in model plants and other legume species. Exploration of sequence data on coding regions is in regular use for the identification of genes in different plant species such as *Medicago*, wheat, barley, tomato, potato, pine, sunflower and many others **[5, 6].** Environmental factors such as drought, fluctuating temperatures, high salinity can affect plant growth and performance and in the case of agronomical important crops, this may translate into reduced yield **[7].** Characterization and cloning of plant genes that confer abiotic stress tolerance thus remains an important exercise in modern plant research.

The present study focused on the identification of abiotic stress responsive genes in chickpea based on sequence similarity approach using previously identified abiotic stress responsive genes, in model plants like *Arabidopsis* and rice and other legume crops including *Medicago truncatula* and *Glycine max*. During present study, ten abiotic stress responsive genes have been identified in chickpea on the basis of sequence similarity. These candidate genes will help in overcoming various abiotic stress related problems limiting chickpea production and subsequently in marker-assisted breeding of superior varieties that survive and give better yield under different abiotic stresses.

Methodology:

Plant material and DNA extraction

Eight diverse chickpea genotypes namely Avrodhi, ICCV 2, ICCV 10, L 550, Pusa 256, Pusa 362, Pusa 391 and Pusa 1103 obtained from Division of Genetics, Indian Agricultural Research Institute, New Delhi were used for the identification of abiotic stress responsive candidate genes. Total genomic DNA was extracted from leaves of 2-week old seedlings using a modified CTAB method **[8].** The quality and quantity of extracted DNA was checked on 0.8% agarose gel.

Identification of abiotic stress responsive genes

Sequence similarity approach was adopted for the identification of stress responsive genes. Ten known candidate genes were selected on the basis of prior information about their involvement in drought tolerance mechanism in other crop species **Table 1 (see supplementary material)**. ESTs available in NCBI EST database (dbEST- http://www.ncbi.nlm.nih.gov/dbEST/) for abiotic stresses, were used for contig assembly after eliminating redundancy using sequence assembly program *CAP3* **[9].** These contigs were then used for similarity search against known candidate genes for abiotic stress tolerance. Unigene sequences showing significant match with a candidate gene were selected and used for primer designing using primer3 software **Table 2 (see supplementary material).**

PCR and confirmation of candidate genes

PCR amplification for all 10 genes mentioned in **Table 2 (see supplementary material)** was standardized and carried out for eight diverse chickpea cultivars in Eppendorf gradient thermal cycler in 10 µl reaction containing 20 ng template DNA, 0.2 mM dNTPs, 2 mM $MgCl_2$, 10 pmol of each of forward and reverse primer, 0.5 U of *Taq* DNA polymerase (SafLabs Pvt. Ltd.), and 1X PCR buffer. The amplification profile included: initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation for 40 s at 94°C, annealing at temperature specific for each target gene for 40 s and extension at 72°C for 1 min 20 s. Final extension was allowed for 10 min at 72°C and storage at 4°C. Amplified PCR products were resolved on 1.2% agarose gels.

PCR products were gel extracted using QIAquick gel extraction kit (Qiagen, USA). Quality and quantity of eluted samples was checked on 1.2% agarose gel. Sequencing of eluted samples was performed on MegaBACE 500 automated DNA sequencer (GE Healthcare) using DYEnamic Dye terminator kit (GE Healthcare). Sequencing PCR was carried out in 10 µl reactions containing template DNA 50 ng, sequencing premix (8 µl), 5 pmol of any of the forward and reverse primer in separate reactions. Sequencing PCR was carried out using Eppendorf thermal cycler and amplification regime included 35 cycles of denaturation at 95°C for 15 s followed by annealing at Tm-5°C for 15 s and extension at 60°C for 2 min and left at 4°C till further processing. Sequencing PCR products were precipitated in 96 well PCR plate using precipitation solution provided with sequencing kit. Sequence data was processed for base calling

and assembled using *Phred* and *CAP3* software's, respectively **[9]**. Good quality sequences generated from *CAP3* were used for similarity search against the corresponding target gene using BLASTN and BLASTX. BLAST results confirmed the presence

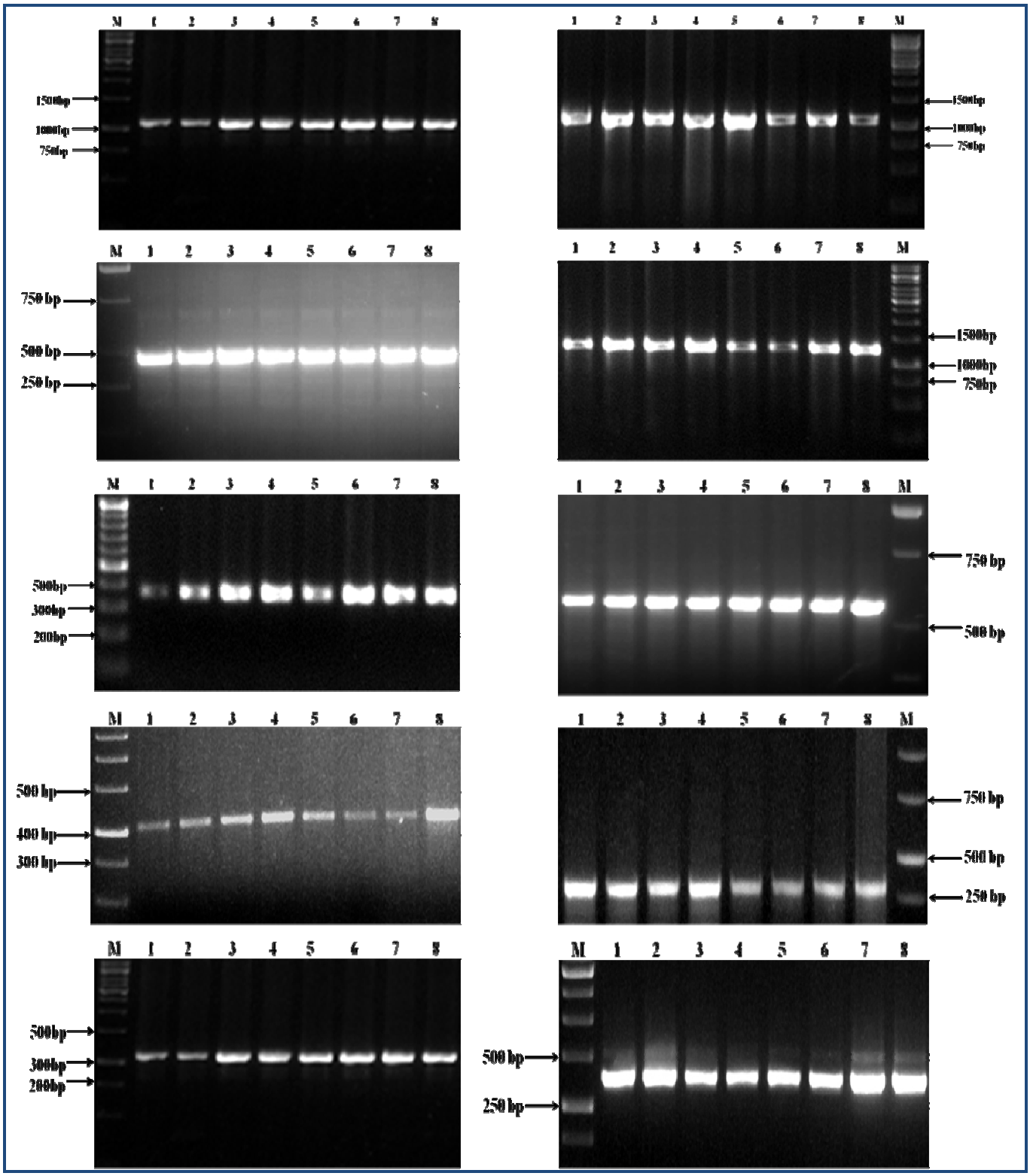

Figure 1: PCR amplification of ten abiotic stress responsive genes from eight diverse chickpea genotypes a. *AKIN*; b. *AMADH*; c. *DHN*; d. *DREB*; e. *Myb*; f. *CAD*; g. *EREBP*; h. *LEA*; i. *SAMS*; j. *STPK*

Result and Discussion:

EST sequences available at NCBI EST database were used for the identification of abiotic stress responsive genes. A total of ~24,700 chickpea ESTs were downloaded from NCBI database. These EST database sequences show high sequence redundancy. Using CAP3 software, these 24,700 EST sequences were assembled into 3,236 contigs and about 5,300 singletons. A total of 8,536 unique long coding sequences were then used for the identification of abiotic stress responsive genes using sequence similarity approach. Chickpea homologues of these abiotic stress responsive genes were identified by sequence similarity search BLAST (BLASTN and BLASTX) from these chickpea coding sequences.

All ten abiotic stress responsive candidate genes were partially amplified in eight diverse chickpea cultivars using gene specific primer pairs. Researchers have isolated the *AKIN* homologues in various plant species including *Arabidopsis*, rice, potato and tobacco and established their role in abiotic stress response **[10]**. *AKIN* homologue was amplified using *AKIN* specific primer pair designed considering unigene sequence showing match with *Arabidopsis AKIN* (SNF-1 related protein kinase). Approximate amplicon size of *AKIN* was ~1100 bp **(Figure 1a)**. Amplification of *AMADH* (Aminoaldegyde dehydrogenase) homologue yielded a product of ~1200 bp **(Figure 1b)**. Protective/curative role of *AMADH* gene in response to stress events caused by mechanical injury has been reported earlier in pea seedlings **[11].** Dehydrin homologue was amplified using primer pair designed for known dehydrin gene using chickpea unigene. Approximate amplicon size of Dehydrin (DHN) gene was ~380 bp **(Figure 1c)**. *DHNs* are one of several proteins that have been specifically associated with qualitative and quantitative changes in cold hardiness **[12]**. *Arabidopsis* plants engineered for *DHN* over-expression, showed improved survival when exposed to low temperature **[13].** *DREB* (Dehydration response element binding) homologue in chickpea was also amplified using primer pairs designed using unigene showing match against *DREB* gene. Approximate amplicon size of the *DREB* gene was ~1200 bp **(Figure 1d)**. Researchers have shown the role of chickpea *DREB2* homologue in plant-growth development and abiotic stress response pathway using transgenic approach **[14]** and isolated *DREB2A* homologue in rice, barley, sorghum and legumes using specific or degenerate primers **[15].** About 350 bp long *MYB* genes was amplified using unigene sequence showing match against *Glycine max Myb* transcription factor **(Figure 1e)**. Role of plant Myb-proteins have been well characterized by using different genetic approaches. In most of the cases, the Myb domain binds to a specific DNA sequence (C/TAACG/TG) to facilitate transcriptional activation **[16].** About 500 bp long Cinnamyl alcohol dehydrogenase (*CAD*) gene homologue was isolated from eight chickpea genotypes using primers designed for contig showing match with cinnamyl-alcohol dehydrogenase *(CAD)* gene of *Arabidopsis thaliana* **(Figure 1f)**. *CAD* is expected to play a key role in plant defence against various abiotic and biotic stresses **[17]**. For isolation of ethylene-responsive element binding protein (*EREBP*) gene homologue in chickpea, primers were designed using contig sequence showing similarity against ethylene-responsive transcription factor from *Arabidopsis thaliana*. Amplification carried out across eight chickpea genotypes produced about 400 bp amplicons **(Figure 1g)**. The *AP2/EREBP* genes play various roles in developmental processes and in stress-related responses in plants. Late embryogenesis abundant *(LEA)* genes represent a gene family that plays important role in vegetative tissues in response to drought, salinity, cold stress and exogenous application of abscisic acid **[18]**. Primers designed using contig showing sequence similarity with *LEA* domain-containing protein *Arabidopsis thaliana* were used to isolate late embryogenesis abundant *(LEA)* gene in chickpea. Amplicons across eight genotypes in chickpea yielded products of about 600 bp **(Figure 1h)**. Expression analysis of the *SAMS* genes during ovary senescence and fruit development showed that *SAMS* transcript levels were up-regulated by auxins during fruit setting and by ethylene during ovary senescence **[19].** For isolation of *S-adenosylmethionine synthetase1* gene homologue in chickpea, primers were designed using contig sequence showing similarity against *S-adenosylmethionine synthetase 1* (*SAM1*) gene of *Arabidopsis thaliana*. PCR amplification yielded about 300 bp amplicons across eight chickpea genotypes **(Figure 1i)**. Serine/threonine protein kinase (*STPK*) gene homologue was amplified using the STPK specific primer pair designed considering unigene sequence having similarity with *Arabidopsis thaliana* putative serine/threonine protein kinase. Amplicon size of *STPK* gene was approximately 450 bp **(Figure 1j)**. *STPK* have been shown to play an important role in response to abiotic stress response and seed development in peanut **[20].**These PCR amplicons were purified and sequenced from both ends. These forward and reverse sequences were used for the construction of single longer sequence, later used for similarity search using BLAST tool (BLASTN and BLASTX) to confirm the presence of chickpea homologues of these genes **Table 3 (see supplementary material).**

Conclusion:

Present study was undertaken with an objective to identify abiotic stress responsive genes in chickpea. Ten abiotic stress responsive candidate genes previously validated for their significance in stress responses in various model crops and other legumes, were amplified in chickpea and sequenced after purification using gene specific primer pairs. PCR amplicons were directly used for DNA sequencing after purification using gene specific primers. Good quality sequences were then used for confirmation of these genes in chickpea using sequence similarity approach based on sequence information of these genes from related crop species or model plants. Therefore, present study provides basic information about some abiotic stress responsive genes in chickpea that can be exploited in overcoming various abiotic stress related problems limiting chickpea production and subsequently in breeding superior varieties giving better yield under abiotic stresses conditions by pyramiding favorable alleles for stress response in single variety using modern molecular breeding approaches.

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Supplementary material:

Table 1: List of selected abiotic stress responsive genes based on literature survey

Gene	Function	Mechanism of action	Reference
DREB	Dehydration responsive	Regulates expression of genes that encode RNA-binding proteins, sugar transport proteins, desaturase, carbohydrate metabolismrelated proteins	[14]
Dehydrin *(DHN)*	Response to water stress	Prevent structural damage and maintain the activity of essential enzymes by interacting with and stabilizes membranes and macromolecules	[21]
STPK	Drought stress	ATP-dependent phosphorylation	[20]
CAD	Response to salt stress	Tissue-specific developmental lignification	[17]
AMADH	Wound healing, abiotic stress responsive	Role in physiological processes related to polyamine degradation	[22]
EREBP	Ethylene responsive	Regulating the expression of PR genes	[23]
LEA Gene	Response to water stress	Accumulate is related to the seed desiccation process	[18]
AKIN	Positive regulator of drought tolerance	Controls many basic processes like glycogen storage, sporulation and thermo-tolerance	[10]
Myb transcription factor	Transcription factor involved in response to salt stress	Facilitate transcriptional activation	[16]
SAMS	Response to cold	Play a key role in ethylene biosynthesis	[24]

AKIN: SNF-1 related protein kinase; *AMADH*: Amino aldehyde dehydrogenase; *CAD*: Cinnamyl alcohol dehydrogenase; *DREB*: Dehydration responsive element binding protein; *STPK*: Serine threonine protein kinase; *EREBP*: Ethylene responsive element binding protein; *SAMS*: S-adenosine methionine synthase

Table 2: List of primers used to amplify chickpea candidate genes related to abiotic stress response

S. No	Gene	Primer Sequence (5`-3`)
1	*DREB*	F: CTT CAT TCG ATC CAG ATT CGG; R: AAC GCG AGT TTT CAG GCC CT
2	Dehydrin *(DHN)*	F: AAA GTG GTG TTG GGA TGA CC; R: TCC TCT CTC CCG AAT TCT TG
3	*STPK*	F: TGG ACA TAT GGG CAC TTG GG; R: TGG ACC AGA AAG CAC CAA GC
4	*CAD*	F: GAA GTG TGC CGA CGA TAA GC; R: GGC TTC AGA AAC CAA GTC ACC
5	*AMADH*	F: TTG GAA GAA GGT TGC AGG CTA G; R: CCC ATT CTC CCA GTT CAC GG
6	*EREBP*	F: ACC GAG GAA AGA TGC CAA TG; R: ATC CAT GCT TTC TGC TGC TG
7	*LEA* Gene	F : GTG GTT GGG CTA TTG CTG TG; R : TCG TTG TCC ACA ACC TCC AC
8	*AKIN*	F : GTG GTT CAG GTG CAG ACT TG; R : TCA GAA AGT GCC CAT CAC GC
9	Myb transcription factor	F : ATG CTA CTG CTG CCT ACA AG; R : ACC GCA GTA CAC TCC AAG AG
10	*SAMS*	F : ACC TCC CAT GTG AAA TCA GC; R : GGA GTG GTG CTT ACA TTG TG

AMADH: Amino aldehyde dehydrogenase; *CAD*: Cinnamyl alcohol dehydrogenase; *DREB*: Dehydration responsive element binding protein; *STPK*: Serine threonine protein kinase; *EREBP*: Ethylene responsive element binding protein; *AKIN*: SNF-1 related protein kinase; *SAMS*: S-adenosine methionine synthase

Table 3: Summary of sequences generated and confirmation of genes in chickpea based on sequence similarity using BLASTN and BLASTX

Gene	Amplicon size (bp)	BlastN result	Accession No.	e-value	BlastX result	Accession No.	e-value
AKIN	795	SNF1-related protein kinase catalytic subunit alpha KIN10-like [*Glycine max*]	XM_003552497.1	2.00E-71	Ser/Thr protein kinase [*Lotus japonicus*]	BAD95888.1	3.00E-45
		SNF1-related protein kinase (AKin10) gene [*Arabidopsis thaliana*]	M93023.1	2.00E-69	SNF1-related protein kinase catalytic subunit alpha KIN10 [*Arabidopsis thaliana*]	NP_850488.1	2.00E-44
AMADH	992	Aminoaldehyde dehydrogenase (amadh2 gene) [*Pisum sativum*]	AJ315853.2	4.00E-53	Aminoaldehyde dehydrogenase 2 [*Solanum lycopersicum*]	NP_001234235.1	1.00E-36
		Betaine aldehyde dehydrogenase 1 (ALDH10A8) [*Arabidopsis thaliana*]	NM_106150.3	2.00E-33	Aminoaldehyde dehydrogenase 1b [*Zea mays* subsp. mays]	AEP68091.1	7.00E-34
CAD	402	Cinnamyl alcohol dehydrogenase-like mRNA [*Glycyrrhiza uralensis*]	EF571301.1	8.00E-43	Cinnamyl alcohol dehydrogenase [*Pyrus pyrifolia*]	AEN94093.1	3.00E-09
					Putative cinnamyl-alcohol dehydrogenase [*Arabidopsis thaliana*]	NP_175552.2	5.00E-06
DHN	388	Dehydrin 1 [*Cicer pinnatifidum*]	AY170010.1	3.00E-66	Dehydrin 1 [*Cicer pinnatifidum*]	AAN77521.1	7.00E-21
DREB	785	Dehydration responsive element binding protein (CAP2) [*Cicer arietinum*]	DQ321719.1	9.00E-94	Dehydration-responsive element-binding protein 2A-like [*Glycine max*]	XP_003539883.1	6.00E-09
					Dehydration responsive factor [*Medicago truncatula*]	XP_003597059.1	0.001
EREBP	335	Putative transcription factor EREBP [*Trifolium pratense*]	AB236754.1	8.00E-71	Ethylene-responsive transcription factor [*Medicago truncatula*]	XP_003627419.1	1.00E-40
		Ethylene-responsive transcription factor RAP2-2 (RAP2.2) [*Arabidopsis thaliana*]	NM_112281.2	0.019	Ethylene-responsive transcription factor RAP2-12 [*Arabidopsis thaliana*]	NP_175794.1	3.00E-08
LEA	501	Late embryogenesis abundant domain-containing protein (AT1G52690) [*Arabidopsis thaliana*]	NM_104147.2	6.00E-05	Late embryogenesis abundant protein 2-like [*Glycine max*]	XP_003529813.1	1.00E-04
		Putative CapLEA-2-like protein (LEAa2-O) gene [*Trifolium repens*]	EU846206.1	4.00E-80	Late embryogenesis abundant domain-containing protein [*Arabidopsis thaliana*]	NP_188188.1	0.001
MYB	330	MYB transcription factor MYB93 (MYB93) [*Glycine max*]	NM_001250591.1	4.00E-39	MYB transcription factor MYB93 [*Glycine max*]	NP_001237520.1	1.00E-25
		Myb-like transcription factor family protein (AT5G47390) [*Arabidopsis thaliana*]	NM_124110.3	0.23	Myb-like transcription factor family protein [*Arabidopsis thaliana*]	NP_199550.1	4.00E-06
SAMS	271	S-adenosylmethionine synthetase (MTR_4g123810) [*Medicago truncatula*]	XM_003609813.1	3.00E-84	S-adenosylmethionine synthase-like isoform 1 [*Glycine max*]	XP_003546550.1	3.00E-57
		S-adenosylmethionine synthetase 1 (SAM1) [*Arabidopsis thaliana*]	NM_100131.2	1.00E-79	Putative S-adenosyl methionine synthetase [*Oryza sativa* Japonica Group]	NP_001042349.1	7.00E-57
STPK	408	Ser/Thr protein kinase [*Lotus japonicus*]	AB184974.1	1.00E-119	Ser/Thr protein kinase [*Lotus japonicus*]	BAD95980.1	1.00E-26
		Putative serine/threonine protein kinase (At2g32850) [*Arabidopsis thaliana*]	BT001103.1	2.00E-34	Putative serine/threonine protein kinase [*Arabidopsis thaliana*]	NP_565756.1	8.00E-20