Identification of genes associated with stress tolerance in moth bean [*Vigna aconitifolia* (Jacq.) Marechal], a stress hardy crop

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Abstract Moth bean is the most drought and heat tolerant cultigens among Asian *Vigna*. We performed comparative transcriptome analysis of moth bean cultivar “Marumoth” under control and stress condition. De novo transcriptome assembly was carried out by using Velvet followed by Oases softwares. Differential expression analyses, SSR identification and validation and mapping of pathways and transcription factors were conducted. A total of 179,979 and 201,888 reads were generated on Roche 454 platform and 48,617,205 and 45,449,053 reads were generated on ABI Solid platform for the control and stressed samples. Combined assembly from Roche and ABI Solid platforms generated 16,090 and 15,096 transcripts for control and stressed samples. We found 1287 SSRs and 5606 transcripts involved in 179 pathways. The 55 transcription factor families represented 19.42% of total mothbean transcripts. In expression profiling, ten transcripts were found to be up-regulated and 41 down-regulated while 490 showed no major change under moisture stress condition. Stress inducible genes like Catalase, Cyt P450 monooxygenase, heat shock proteins (HSP 90 and HSP 70), oxidoreductase, protein kinases, dehydration responsive protein (DRP), universal stress protein and ferridoxin NADH oxidoreductase genes were up-regulated in stressed sample. Genes which might be involved in moisture stress

tolerance in moth bean were identified and these might be useful for stress tolerance breeding in moth bean and other related crops.

Keywords Moth bean · Moisture stress · Transcriptome · RNA-Seq · Stress responsive genes

Introduction

Moth bean [*Vigna aconitifolia* (Jacq.) Marechal] belonging to the family Fabaceae, is a drought and heat tolerant crop grown throughout the tropical and sub-tropical regions of the world. Despite its multiple uses as food, feed, medicine and cosmetics; cultivation of moth bean is restricted to marginal lands under rainfed conditions. Moth bean with short internodes and hairy branches grows to a height of 15–30 cm. Due to its harsh natural habitat, moth bean has evolved a few morphological and physiological features that impart tolerance to extreme environmental conditions (Kumar and Singh 1988). Moth bean is a herbaceous, short day crop having deep and fast penetrating root system, broad canopy, winy and semi-trailing growth habits. Because of these adaptive features, it can survive under drought stress and heat stress conditions. The impeding effect of climate change on growth and productivity of crop plants re-emphasizes the importance of understanding the series of cellular, biochemical and molecular processes that enable plants to tolerate abiotic stresses such as heat stress, moisture stress, salinity and extreme cold. Crops such as moth bean that are resistant or tolerant to these abiotic stresses are important biological systems to study the resistance mechanisms. Due to its short life cycle and small genome size, moth bean has the potential to be used as model plant for such genetic studies. Identification of genes

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involved in stress tolerance requires extensive information on regulation of gene expression as well as biochemical function of individual proteins involved in tolerance mechanism. In recent years, high throughput next generation sequencing (NGS) platforms like Applied Biosystems SOLiD, Illumina Miseq and HiSeq, Roche 454 and Ion Torrent have been used extensively to generate genomic and transcriptome resources. Unlike genome sequence, transcriptome sequence is a direct indication of gene expression levels. For those species whose genomes have not been sequenced, *de novo* transcriptome assembly is becoming a common commercial (Zhai et al. 2014; Li et al. 2010, Duan et al. 2012; Lu et al. 2010) and powerful approach with high resolution. Transcriptome data mining is an efficient way to discover genes or gene families encoding enzymes involved in various important metabolic pathways (Wang et al. 2009; Varshney et al. 2009; Haas et al. 2013). In recent years, transcriptome analysis has been widely used for gene expression studies, discovering novel transcripts and finding differentially expressed genes (Garg et al. 2011; Li et al. 2013; Wenping et al. 2011). EST-SSRs are located in the coding region of the genome with high rate of transferability to related species. A large number of transcriptome studies have been conducted in many crop species in order to understand physiological basis of gene expression (Gupta et al. 1996; Varshney et al. 2005). Further, Gurjar et al. (2014) reported ESTs through suppression subtraction hybridization in moth bean. Genic SSR markers could be used for a variety of applications such as molecular mapping and genetic diversity analysis (Varshney et al. 2005). Considering the importance of this drought hardy crop in the changing climate scenario, this study was devised with an aim to identify genes related to stress tolerance and the regulatory factors expressed under control and moisture stressed conditions in Marumoth, a stress tolerant cultivar of moth bean. Gene expression analysis and functional classification has provided information on different stress responsive genes, transcription factors and signalling genes possibly associated with moisture stress in moth bean, thereby providing an insight into the molecular mechanism involved in drought tolerance.

Materials and methods

Sample preparation and transcriptome sequencing

Marumoth, the cultivar of moth bean used for this study was selected for moisture stress tolerance after an initial screening of a selected set of 240 accessions representing cultigens from all moth bean cultivating regions of India. The control plants were irrigated regularly (once in

7 days). In the moisture stressed plants, water was withdrawn 30 days after germination. Leaves were sampled from the control and treated plants 25 days after withdrawal of water from the treated plants when the soil moisture content was 20%. The soil moisture content was measured using soil moisture monitoring systems (Data-flow Systems Pty Ltd, New Zealand) at 45 cm depth. The total RNA was isolated from the control and stressed samples by using PureLink RNA Isolation Kit (Invitrogen) according to the manufacturer's protocol. The RNA content was determined using a low volume UV–VIS spectrophotometer (NanoDrop ND-1000 spectrophotometer, Thermo Scientific, USA) and assessed for integrity on Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). mRNA libraries were prepared using Rapid Library Prep kit (Roche) and SOLiD Total RNA Seq kit as per manufacturer's protocol and sequenced on Roche 454 GS FLX and ABI SOLiD platforms respectively. Two systems were used in order to achieve better coverage at lower costs.

De novo assembly and functional annotation

High quality transcriptome reads from the control and stressed samples were assembled *de novo* using Velvet version 1.0.13 (Zerbino and Birney 2008) followed by Oases version 0.1.16 (Schulz et al. 2012). *De novo* assembly was carried out using different k-mer sizes (Surget-Groba and Montoya-Burgos 2010) for each sample. Parallel, *de novo* assembly was also run on CLC Genomics Workbench (Fig. 1). Finally, all the contigs generated by using Velvet/Oases Pipeline, CLC Genomics Workbench and the assembled transcripts for Roche 454 GS FLX data (assembled using Newbler and i-Assembler) were merged to generate super transcripts using CAP3 Assembler resulting in high quality and larger contigs. BLASTX alignment against the databases non-redundant proteins (NR), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Cluster of Orthologous Groups (COG) was performed, and the best aligning results were selected on the basis of Expect-value (E-value) of 10^{-5} . BLAST2GO program was used for gene ontology (GO) annotations of assembled transcripts for describing biological processes, molecular functions, cellular components categories (Conesa et al. 2005), and functional classifications were assigned using WEGO software (Ye et al. 2006). The annotated transcripts were further used for identification of stress responsive transcripts. The stress related genes were obtained from online plant stress Gene Database (<http://cbb.jnu.ac.in/stressgenes/>). This list was updated as per the new gene records in National Centre for Biotechnology Information (NCBI) and was used for Stress Responsive Gene Identification. A list of 764 and 538 stress responsive transcripts were identified from control and stressed

Fig. 1 An overview of *de novo* assembly process by using different Kmer sizes

sample, respectively. For identification of transcription factor families in moth bean, the transcripts were searched against all the transcription factor protein sequences in Plant Transcription Factor database (Jin et al. 2014) using BLASTX with an E-value of $\leq 1e-5$.

Read mapping and transcript abundance (expression) measurement

FPKM (Fragments per kilobase of exon per million fragments mapped) values were obtained by conducting RNA-seq experiment for all transcripts from both the samples by mapping onto the assembled transcriptomes (control + stress). Differential expression was ascertained for stressed condition by comparing the FPKM values of similar transcripts under stressed condition in relation to

control. To evaluate the genome wide response of moth bean under control and stressed condition, expression heat map was generated by using MeV (Multi-experiment Viewer) software. Hierarchical clustering was done by using Euclidean distance matrix with an average linkage method using MeV.

EST-SSR search, primer design and validation

MISA (MicroSATellite; <http://pgrc.ipk-gatersleben.de/misa>) was used for SSR mining in moth bean. The minimum number of repeats used for choosing the SSRs was six for di-nucleotide repeats and four for tetra-, penta-, and hexa-nucleotide repeats. BatchPrimer3 software was used for Primers designing with the following criteria: distance between two SSRs was 100 bp, GC content of 45–60%,

annealing temperature of 50–60 °C and PCR product size of 250–300 bp. A random set of 22 EST-SSRs (expressed sequence tags Simple-sequence repeats) markers were validated with 48 moth bean accessions (Table S4 and Table S5).

Results

De novo assembly of sequencing data

The transcriptome from the leaf tissue of the genotype Marumoth was analysed under control and moisture stress conditions using the Roche 454 and ABI Solid sequencing platforms. A total of 179,979 and 201,888 reads were generated on Roche 454 platform and 48,617,205 and 45,449,053 reads were generated on ABI Solid platform, for the control and stressed samples respectively. N50 length was 419 and 238 bp for control and stress samples respectively. The sequence data of both the platforms (Roche 454 and ABI Solid) have been deposited in the Sequence Read Archive (SRA) database for both control and water stress conditions at NCBI under the accession number SRP032962. Following combined assembly from both the platforms (Roche and ABI Solid), 16,090 and 15,096 transcript contigs were generated for control and stressed samples (Fig. 2).

Functional annotation of assembled transcripts

All the assembled high quality transcripts were annotated using BLASTX against NCBI non redundant protein (nr) database with an E value threshold of 10^{-5} . Of the 16,090 and 15,096 transcripts generated for control and stressed

samples, 12,808 and 9687 transcripts were functionally annotated from both the samples.

GO assignments and COG classification

Based on the BLASTX results against the NR database, Blast2GO program was used to obtain Gene Ontology (GO) functional annotations and categorizations of the annotations. GO classification was obtained for 18,362 transcripts out of a total of 22,495 annotated transcripts. GO terms were assigned to the resultant 18,362 unique sequences and classified into 32 GO classes including 15 biological processes, 9 cellular components and 8 molecular functions (Fig. 3). Under the biological process category, metabolic process (13,263) cellular process (10,368 sequences) and cellular component organization (2714) were prominently represented. Within the cellular components, cell (13,401), cell part (13,304) and organelle (8438) were the most highly represented categories. For the molecular function category, the largest proportion of genes were clustered into catalytic activity (10,014), binding (9309), and structural molecule activity (1269). Additionally, few sequences were assigned to death multi organism processes, molecular transducer activity and Enzyme regulator activity categories with less than 300 sequences.

To further predict and classify the possible functions of moth bean transcripts, these unique sequences from moth bean leaf transcriptome were aligned to the COG database. The results showed that 6779 transcripts (21.73% of all transcripts) were assigned to 23 COG categories (Fig. 4). Of these, the largest category was translation, ribosomal structure and biogenesis (1158 sequences), followed by post translational modification, protein turnover,

Fig. 2 Plot shows Marumoth combined assembly in control and stressed samples

Fig. 3 Classification of Gene Ontology (GO) terms in the moth bean transcripts under biological process, molecular function and cellular component categories

Fig. 4 COG functional classification of transcripts

chaperones (968), general function prediction only (819), energy production and conversion (683) and carbohydrate transport and metabolism (515); whereas the clusters of cell motility (5) and nuclear structure (3) represented the smallest classifications.

KEGG pathway annotation

Metabolic pathway prediction in *Vigna aconitifolia* was carried out by annotating the assembled transcripts with the corresponding enzyme commission (EC) numbers in the KEGG database using KAAS (KEGG automatic annotation server) analysis tool (Kanehisa and Goto 2000) against dicot plants as reference. The bidirectional best hit method was used to obtain KEGG Orthology (KO) assignment. A

total of 31,186 KEGG classification was found for 2648 transcripts and classified into 181 different pathways (Table S1) corresponding to several KEGG modules; amongst these pathways, metabolism pathways were the most abundant group (8.57%), with most of them involved in energy metabolism (40.3%) and carbohydrate metabolism (23.7%). Ribosome and oxidative phosphorylation were the highly enriched categories, which were represented by 8.5 and 4.4% of the total KEGG annotated transcripts respectively.

Identification of transcription factors

Moth bean transcripts were searched against Plant TFDB (Jin et al. 2014) using BLASTX to identify the putative

transcription factors. A total of 6058 transcripts (with an e -value ≤ -5) matched in TFDB corresponding to 55 TF families representing 19.42% of total moth bean transcripts (Table S2). Most abundant TF family was basic helix loop helix (bHLH 8.76%), followed by Cys 3 His Zinc finger domain (C3H 7.69%), Ethylene responsive factor (ERF 6.43%), Myeloblastosis (MYB related 6.43%), C2H2 (6.0%), tri-helix (5.95%), NAC (4.15%) and WRKY (2.24%) while RAV and Growth-regulating factors (GRF) were the least abundant TF families (Fig. 5).

Differential expression analysis of assembled transcripts

Gene expression analysis of 16,090 (control) and 15,096 (stress) transcript contigs was carried out by mapping all high quality reads on assembled transcript contigs and FPKM (Fragments Per Kilobase of Exon per Million Fragments mapped) values were calculated. The FPKM values obtained by RNA-Seq were categorized into different categories depending on their abundance (Table 1) since expression analysis can provide important clues about the gene functions. In order to know the differential expression, heat map was generated by using $\log_2$ FPKM expression values. To identify differentially expressed genes, we screened genes that were found to be highly expressed and had statistically significant changes in expression as compared to control (Fig. 6). On the basis of common annotations, we found 541 transcripts showing differential expression in both the samples. Among those, 10 transcripts were up-regulated and 41 down-regulated in the stressed sample and 490 showed no major change under

Table 1 Expression level of assembled transcripts of mothbean

Description	Control	Stress
FPKM > 1000	93	263
500 < FPKM < 1000	116	126
50 < FPKM < 500	4219	2000
FPKM < 50	11,662	12,707

stress condition. Transcripts were considered significantly differentially expressed (> 2 or < -2 in $\log_2$). Up-regulated transcripts under stress conditions included catalase, Cyt P450 monooxygenase, HSP 90, HSP 70, oxidoreductase, protein kinases, dehydration responsive protein (DRP), universal stress protein (USPs) and ferridoxin-NADH oxidoreductase.

Frequency and distribution of different SSR types

Among the 31,186 transcripts generated in the study, 1287 SSR motifs were identified (Table S3). A total of 1161 transcripts contained SSRs. About 107 sequences had more than one SSR and 103 (Table 2) were compound type SSRs. It was observed that tri-nucleotide repeat motifs (846 or 65.7%) were the most abundant, followed by di- (279 or 21.6%), tetra- (83 or 6.4%), penta- (67 or 5.2%) and hexa-nucleotide (6 or 0.9%) repeat motifs (Fig. 7a). The number of SSR repeats ranged from 4 to 34, with four repeats being the most abundant, followed by six and five repeats as the next most abundant. Motifs with more than 16 repeats were rare. Among the dinucleotide repeats, AG/TC was the most abundant (48.7%). The other three major motifs were

Fig. 5 Identification of transcription factor families and their abundance. Top 20 transcription factor families found in moth bean transcriptome based on BLAST results from PlnTFDB database

Fig. 6 Heatmap showing differential expression of moth bean transcripts. The unique transcripts annotations have been given to the right side. The colour scale represents $\log_2$ FPKM values

Table 2 Statistics of SSRs identified in moth bean transcripts

SSR mining	
Total number of sequence examined	31,186
Total size of examined sequences (bp)	9,993,394
Total number of identified SSRs	1287
Number of SSRs containing sequences	1161
Number of sequences containing more than one SSR	107
Number of SSRs present in compound formation	103

GAA/CTT (13%), AGA/TCT (6.85%), and AT/TA (20.4%) (Fig. 7b).

Development of polymorphic genic SSR markers

A total of twenty-two SSR loci were randomly selected (Table. S4) to test for amplification and informative nature by analyzing on a germplasm panel of 48 moth bean accessions (Table. S5). Of the 22 loci tested, 14 produced clear amplicons of the expected size, two amplified non-specific products and six failed to amplify the DNA product. Of the loci successful amplified, polymorphism was detected at only one locus (Fig. 1s and 2s) corresponding to the trinucleotide motif (TGC).

Fig. 7 Distribution of SSR repeat types and repeat motifs. **a** Different repeat type SSRs and **b** different repeat motifs and percentage of transcripts corresponding to them

Discussion

Moth bean (*Vigna aconitifolia* (Jacq.) Marechal) is a drought and heat tolerant grain-legume grown in arid regions. The ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR) in New Delhi, India, has more than 1000 accessions of moth bean collected from more than eight states of India. Cultivars such as RMO-40, RMO-225 and Marumoth are mostly commonly cultivated varieties in India (Gupta et al. 2016). To develop molecular markers, transcriptome sequencing and de novo assembly is an alternate method to non-genic SSR marker identification in both model and non-model organisms. De novo sequencing is specifically an effective approach for genome analysis without reference genome (Zheng et al. 2013). In this study, Roche 454 and ABI Solid sequencing platform provided high-throughput data and helped in identification of quality EST-SSRs for moth bean at a low cost. Functional annotation of moth bean transcriptome showed that the highly represented terms in the GO, KEGG and COG databases were also identified in addition to transcripts related to drought tolerance (S1). Catalase is the first line of defence against free radicals at cellular level. Therefore, their regulation depends upon the oxidant status of the plant cell. Catalase eliminates H_2O_2 by breaking H_2O_2 to form water and oxygen, thereby reducing toxicity (Gong et al. 2005). The present study revealed that catalase and cytochrome P450 monooxygenase activity were found to be up-regulated in stress as compared to control condition. Earlier studies had reported increased catalase activity in tolerant plants as compared to the susceptible ones (Raheleh et al. 2012). Catalase activity showed a significant increase in leaves of drought-stressed plants (Sofa et al. 2015). Further, in *Brassica napus* higher level of catalase activity was reported under drought stress conditions, thereby indicating that such plants may perform better under drought stress (Abedi and Pakniyat 2010).

Cytochrome P450 monooxygenases (P450 s) play crucial role in the synthesis of lignin, secondary metabolites, fatty acids, hormones, and signalling molecules in all plants (Werck-Reichhart et al. 2002; Kroetz and Zeldin 2002). Cytochrome P450 s influence a variety of metabolic pathways. In soybean, the expression of *GmCYP82A3* (from soybean *CYP82* gene) can be induced by various abiotic stresses and phytohormones molecules (Yan et al. 2016). Further, in *Arabidopsis thaliana*, expression of *CYP709B3* was shown to be induced by salt stress and it plays a role in regulation of salt tolerance (Mao et al. 2013). The role of heat shock protein (HSP) during abiotic stress is also well documented (Wang et al. 2004; Song et al. 2014). HSPs are responsible for protein folding and can help in protein refolding under stress conditions (Buchner 1999; Young et al. 2001; Pratt and Toft 2003) and it was reported that the main role of Hsp90 is to manage protein folding, cell-cycle control, protein degradation, protein trafficking and signal-transduction. A recent study showed that Hsp90 interacts with the 26s proteasome and plays an important role in its assembly and maintenance (Imai et al. 2003). In the present study, we have detected overexpression of heat shock proteins (HSPs70 and HSPs 90) which play key roles in response to drought stress as well as heat stress (Fig. 6). Universal stress proteins (USPs) were also over expressed in stressed condition and helped in the survival of the plants under harsh conditions (Karolina et al. 2013). Earlier studies had demonstrated the prominent role of protein kinases in the regulation of cell differentiation, growth and development of plant system (Ling Ho et al. 2015). Serine/threonine kinase (SnRKs), a type of protein kinase also seems to be crucial in linking stress and metabolic responses, whose role in regulation of plant response to abiotic stress was recently identified. SnRKs are implicated in abiotic stress tolerance such as cold, salt, drought and heat as well as in biotic stress. Serine/threonine protein kinases show higher expression in abiotic stress condition

(Afjal et al. 2008). Overall, this analysis suggested that dehydration responsive proteins (DRPs) play an important role in increasing plant tolerance to environmental stresses such as drought, cold and heat. DRPs were found to be up-regulated under moisture stress condition. In this study, we observed that many genes related to heat stress were also expressed in moisture stress condition. The transferable nature of EST-SSR markers within related species increases its utility in important plant breeding programmes. EST-SSR have been reported to be less polymorphic than genomic SSR in plants since DNA sequences are more conserved in transcribed regions (Varshney et al. 2005). However, there is a need to explore use of EST-SSRs as markers since presence of polymorphism in these regions are likely to indicate corresponding changes in gene expression, thereby indicating their greater utility in trait tagging for use in marker assisted breeding. Similar to the present study, several earlier studies reported the validation of EST SSR markers from the transcriptome data in legumes such as *Vigna radiata*, *V. angularis*, *V. umbellata* and chickpea (Choudhary et al. 2009; Gupta and Gopalakrishna 2010; Chankaew et al. 2014; Chen et al. 2015; Kang et al. 2015). As no genome or transcriptome sequences of moth bean have been reported until now, present findings provide some insight into transcriptomes in moth bean. The study has identified differentially expressed genes and related pathways from control and stress leaves samples of moth bean. De novo assemblies, functional annotations and their characterization with gene ontology and metabolic pathway analysis provided a range of stress-associated genes in moth bean and these findings will contribute to understanding of stress tolerance mechanism in moth bean and other related breeding crops. Transcription factors play crucial role in plant adaptability to abiotic stress at molecular level (Sharma et al. 2013). A total of 6058 transcripts matched in TFDB belong to 55 TF families. Within these bHLH, MYB related, C3H, ERF, NAC and WRKY TF families were found to be abundant in our study. The basic Helix-Loop-Helix (bHLH) proteins are a group of functionally diverse transcription factors found in both plants and animals. Many studies have reported that bHLH-type proteins act as transcriptional regulators and are involved in drought stress response in *Arabidopsis* (Jiang et al. 2009) and in rice the overexpression of OrbHLH001 enhances salt tolerance by altering ion flux in roots (Chen et al. 2013). As one of the largest TF families, MYB transcriptional factor is a multi functional gene family that have been found in response to plant abiotic stress. Among the members of MYB, TaMYB33 shows high identity to R2R3-MYB transcription factor which indicate stress tolerance in rice and maize. TaMYB33 TF was induced by different stresses such as salt, drought and ABA treatments, and its over-expression in

Arabidopsis plant enhanced tolerance to salt and moisture stresses. Hence, the TaMYB33 became a model candidate gene for breeding of wheat for abiotic stress tolerance (Li-Chao et al. 2009; Qin et al. 2012). The regulatory role of WRKY TF family was also confirmed in *Arabidopsis* during salinity stress (Chen et al. 2013).

Conclusion

The present study has provided a comprehensive idea about the transcriptomes in relation to stress tolerance in moth bean. The transcriptomes generated were also helpful in marker development in moth bean. The transcripts identified have been observed to have a role in different metabolic pathways. The results provide genomic resources for understanding stress tolerance mechanisms in moth bean, thereby helping in acceleration of studies in functional genomics and their application in breeding programs. In addition, the SSRs identified in stress-associated genes in this study will be new resources for molecular breeding in moth bean and other related *Vigna* species.

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