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REVIEW ARTICLE

EXPRESSED SEQUENCED TAGS (ESTs) - A Functional Genomic Approach For Gene Discovery

Shabir H. Wani*, N. B. Singh**, Hitesh K. Saini***, L. Promin Devi**, P. Monalisa**

*Biotechnology Laboratory, Central Institute of Temperate Horticulture, Srinagar, (J&K) 190 007 India.
**Department of Plant Breeding and Genetics, COA, Central Agricultural University, Imphal, Manipur, 795 004
***Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana 141 004

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ABSTRACT

Abiotic stresses like drought and high salinity are the most damaging factors to agricultural productivity worldwide. Functional genomics has played a key role in making plant breeding more efficient in development of crop varieties tolerant to biotic and abiotic stresses. Numerous genes and their products respond to these stresses at transcriptional and translational level. Discovery of these novel genes has been the main objective of all plant breeders. Although many drought responsive genes have been discovered, it is still of great importance to analyse drought-inducible genes and their expression in drought-tolerant crops. In addition to various molecular markers, Expressed Sequenced Tags (ESTs) are currently used as a fast and efficient method of profiling genes expressed in various tissues, cell types or developmental stages. The genes, thus, discovered will be utilized in their transfer to commercially important crops through marker assisted selection or transgenic breeding.

INTRODUCTION

Agricultural productivity is severely affected by abiotic stress factors like drought and high salinity (Chen *et al*., 2010a). As a consequence, physiological and biochemical responses in plants differ and cellular ionic balances are disrupted. The productivity and yield of plant crops is often limited by the joint influence of several stress combinations (Mittler, 2006). Identification of genes exhibiting expression responses to several stresses may provide insights into the functional basis of multiple stress tolerance in plants. Numerous genes and their products respond to abiotic stresses at transcriptional and translational level (Cushman and Bohnert, 2000; Sreenivasulu *et al*., 2004; Yamaguchi-Shinozaki and Shinozaki, 2005; Umezawa *et al*., 2006). Although many drought responsive genes have been discovered, it is still of great importance to analyse drought-inducible genes and their expression in drought-tolerant crops (Medini *et al*., 2009). To unravel the possible mechanisms of stress tolerance we need to understand the functions of these stress-inducible genes. Now, functional genomic approaches have led a major paradigm from single gene discovery to thousands of genes by using multiple use and efficient techniques. Molecular approaches for detecting differences in the DNA of individual plants have many applications of value to crop improvement.

***Corresponding author:** N.B.Singh:
e-mail ID: brajendracau@yahoo.co.in

These differences are known as molecular markers because they are often associated with specific genes and act as 'signposts' to those genes. Several types of molecular markers that have been developed and are being used in plants include restriction fragment-length polymorphisms (RFLPs), amplified fragment-length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), cleavable amplified polymorphic sequences (CAPS), single strand conformation polymorphisms (SSCP), sequence-tagged sites (STS), simple sequence repeats (SSRs) or microsatellites, and single-nucleotide polymorphisms (SNPs) (Gosal *et al*., 2009). Such markers, closely linked to genes of interest, can be used to select indirectly for the desirable allele, which represents the simplest form of marker-assisted selection (MAS), now being exploited to accelerate the backcross breeding and to pyramid several desirable alleles (Singh *et al*., 2001). Selection of a marker flanking a gene of interest allows selection for the presence (or absence) of a gene in progeny. Thus molecular markers can be used to follow any number of genes during the breeding program (Paran and Michelmore, 1993). The discovery of molecular markers has enabled dissection of quantitative traits into their single genetic components (Tanksley 1993, Bernier *et al*., 2007, Kato *et al*., 2008) and helped in the selection and pyramiding of QTL alleles through MAS (Ribaut *et al*., 2004; Neeraja *et al*., 2007; Ribaut and Ragot, 2007; Guo *et al*., 2008; Khowaja *et al*., 2009; Chen *et al*., 2010b).

Nowadays scientists are utilizing Expressed sequenced Tags (ESTs) as markers for discovering novel genes. ESTs are short DNA sequences corresponding to a fragment of a complimentary DNA (cDNA) molecule and which may be expressed in a cell at a particular given time. ESTs are currently used as a fast and efficient method of profiling genes expressed in various tissues, cell types or developmental stages (Adams et al., 1991). One of the many interesting applications of ESTs database (dbEST) is gene discovery where many new genes can be discovered. Generation of expressed sequence tags (ESTs) from cDNA libraries prepared from abiotic stress-treated seedlings of various crops, complete genome sequence information of rice and *Arabidopsis* and many other commercially grown crops provided a valuable resource for gene discovery. Furthermore, employment of multi-parallel techniques such as expression profiling by microarrays, random and targeted mutagenesis, complementation and promoter-trapping strategies allow the identification of the key stress-responsive gene pools and in turn provide important clues for functional characterization of stress responsive genes and stress tolerance mechanisms. Genomic studies show considerable overlap of plant responses to osmotic stresses such as drought, and salinity (Chen *et al*., 2002; Kreps *et al*., 2002). The discovery of novel genes and its possible utilization in modern plant breeding has proven to a major step ahead for managing abiotic stress in plants (Gosal *et al*., 2009). Many reports are available that show transgenic plants developed by utilizing the genes identified by different techniques including EST approach, for example *OsglyII* gene introduced into rice for tolerance to salinity stress (Wani *et al*., 2009a, b, ; Wani and Gosal, 2010) and many other similar reports have been reviewed (Wani *et al*., 2008, Gosal *et al*., 2009; Gosal *et al*., 2010). In this review paper advances in gene discovery through EST based approach will be discussed.

IDENTIFICATION OF GENES – EST APPROACH

Functional genomic approaches may provide powerful tools for identifying expressed genes. The discovery of novel genes and its possible utilization in modern plant breeding continue to engage the attention of most plant biologists. ESTs are short DNA molecules (300 - 500 bp) reverse-transcribed from a cellular mRNA population (MacIntosh *et al*., 2001). They are generated by large scale single-pass sequencing of randomly picked cDNA clones and have proven to be efficient and rapid means to identify novel genes (Adams *et al*., 1991). ESTs thus represent informative source of expressed genes and provide a sequence resource that can be exploited for large-scale gene discovery (Whitefield et al., 2002). By using comparative genomic approaches, the putative functions for some of these new cDNA clones may be found (Velculescu *et al*., 1995) and thereby constitute an important tool for a better understanding of plant genome structure, gene expression and function (Lopez *et al*., 2005). A large number of ESTs have been studied and generated from various plant species including both mosses and cycads (Brenner *et al*., 2003; Kirst *et al*., 2003) model and crop plants like *A. thaliana*, rice, wheat and maize and *Medicago trunculata*.and recently in *Brassica napus* (Chen *et al*., 2010).

IDENTIFYING NOVEL ABIOTIC STRESS TOLERANCE GENES

An important genomic approach to identify abiotic stress related genes is based on ESTs generated from different cDNA libraries representing abiotic stress treated tissues collected at various stages of development. Detailed information on the type of libraries and number of ESTs generated from each library of various abiotic stress-tolerant species is indexed at the National Center for Biotechnology Information (NCBI) dbEST (http ://) www. ncbi. nlm. nih. gov/ dbEST/dbEST_summary.html). In order to enrich plant EST datasets with stress-responsive genes, specific sequencing programs based on cDNA libraries from stress-treated plant tissues and organs of diverse species at many developmental time points are necessary. Since EST data sets generated from control as well as stress-treated tissues are derived mostly from non-normalized cDNA libraries, counting the abundance of a particular gene provide information on relative expression levels of stress-responsive genes. In addition, the clustering of EST sequences generated from abiotic stress-treated cDNA libraries provides information on gene number, gene content and possible number of gene families involved in stress responses. Putative functions are assigned to such stress-responsive genes by BLASTX comparison to the Swissprot database. This type of analysis provides a valuable resource of information regarding a gene index associated with stress-responsive genes among various species. Further, the clustering data yields consensus sequences that provide a much cleaner data set than typical EST data. Outcome of such studies indicates that unknown genes still represent a very high percentage (20–30%) in all cDNA libraries of stress treated plants. They need to be annotated in order to find possible functions and to get a comprehensive picture of the tolerance mechanisms. An attempt was made to identify abundantly expressed ESTs in libraries of a salt-treated halophyte *Thellungiella halophila* as well as from monocots like barley, wheat, maize and rice (Sreenivasulu *et al*., 2004b). Analyzing the various EST collections enabled us to find stress-regulated genes. Further, these data should also assist in unraveling the underlying regulatory and metabolic networks. Based on the search results a total of approximately 13,022 abiotic-stress related ESTs were reported from *Hordeum vulgare*, 13,058 from *Oryza sativa*, 2,641 from *Secale cereale*, 17,189 from Sorghum bicolor, 20,846 from *Triticum aestivum* and 5,695 from *Zea mays*. However, the number of ESTs generated so far solely from stress-treated libraries is low, as compared to total ESTs. Therefore, there is a need to enforce sequencing programmes from stress-tolerant genotypes of cereals (treated with different abiotic stresses) covering a wider range of tissue types and developmental stages. ESTs clones assembled into 375 contigs and 696 clusters when *Glycine soya* was subjected to saline conditions with the objective of mining salt tolerance genes. A number of ESTs have been generated and produced by studying genes involved in stress adaptation in the mangrove plant *Acanthus ebracteatus* Vahl (Huang and Madan, 1999), studying the genome of *Panax ginseng* C.A Meyer (Choi *et al*., 2005; Kim *et al*., 2006). Coles *et al*. (2005) developed and characterised an EST database for quinoa (*Chenopodium quinoa* Willd)

and demonstrated the usefulness of EST libraries as a starting point for detecting DNA sequence polymorphisms (SNPs). They compared cDNA sequenc-es of quinoa with sequences in the TIGR *A. thaliana* and GeneBank protein database. 67% of the quinoa proteins showed homology to Arabidopsis proteins with putative function, 18% had no significant matches, 9% had significant homology to *Arabidopsis* proteins with no known function and 6% sharing significant homology with plant proteins from species other than Arabidopsis. According to the dbEST release (September, 2007), there are currently over 46 million ESTs belonging to both plants and animals. Although there is no real substitute for a complete genome sequence, EST sequencing certainly avoids the biggest problems associated with genome size and the accompanying retrotransposon repetitiveness (Tang *et al*., 2003). In the case of chickpea, about 100 expressed-sequence tags (EST) are available in public databases (Boominathan *et al*., 2004). EST sequences from related species such as *Medicago truncatula* allow the identification of genes regulated during nodulation, embryo development and desiccation. In order to identify genes associated with water-stress response in rice, ESTs generated from a normalized cDNA library, constructed from drought-stressed leaf tissue of an indica cultivar, Nagina 22 were used. Analysis of 7794 cDNA sequences led to the identification of 5815 rice ESTs. Of these, 334 exhibited no significant sequence homology with any rice ESTs or full-length cDNAs in public databases, indicating that these transcripts are enriched during drought stress. Analysis of these 5815 ESTs led to the identification of 1677 unique sequences. To characterize this drought transcriptome further and to identify candidate genes associated with the drought-stress response, the rice data were compared with those for abiotic stress-induced sequences obtained from expression profiling studies in Arabidopsis, barley, maize, and rice. This comparative analysis identified 589 putative stress-responsive genes (SRGs) that are shared by these diverse plant species (Gorantla *et al*., 2007). Recently, total, 536 clones were identified to be putative high-salinity- or drought-responsive genes in *Brassica napus*. Among them, 172 and 288 clones are detected to be putative high-salinity- and drought-inducible genes, whereas 141 and 189 are candidates for high-salinity- and drought-suppressed genes, respectively (Chen *et al*., 2010). The functional classification of these genes are indicated that belonged to gene families encoding metabolic enzymes, regulatory factors, components of signal transduction, hormone responses, some abiotic stresses-related proteins, and other processes related to growth and development of B. *napus*. From the upregulated candidate genes, some important genes were further demonstrated to be high-salinity- or/and drought induced expression. In addition to the discovery of abiotic stress tolerant genes an EST data set has been developed for biotic stresses also. For instance, in pigeon pea, a total of 16 cDNA libraries were constructed from four genotypes that were resistant and susceptible to *Fusarium* wilt (FW) and a total of 9,888 (9,468 high quality) ESTs were generated (Raju *et al*., 2010). Clustering and assembly analyses of these ESTs resulted into 4,557 unique sequences (unigenes) including 697 contigs and 3,860 singletons. BLASTN analysis of 4,557 unigenes showed a significant identity with ESTs of different legumes (23.2-60.3%), rice (28.3%), *Arabidopsis* (33.7%) and poplar (35.4%). Therefore it was concluded that pigeonpea ESTs are more closely related to soybean (60.3%) and cowpea ESTs (43.6%) than other plant ESTs.

IDENTIFICATION OF A STRESS-RESPONSIVE GENES BY TRANSCRIPT PROFILING

Insights into gene expression patterns and functions coupled with stress tolerance can be explored by EST-based cDNA arrays. Gene expression profiling using cDNA macroarrays (Sreenivasulu *et al*., 2006b) or microarrays (Chen *et al*., 2002) are novel approaches to identify higher number of transcripts and pathways related to stress tolerance mechanisms than before. There are several studies reported related to abiotic stress transcriptome profiling in model species such as Arabidopsis and rice that have revealed several new stress-related pathways in addition to the previously well described stress-related genes (Kreps *et al*., 2002; Seki *et al*., 2002; Oh *et al*., 2005).The cDNA macro/microarray technology for transcript profiling has been established, based on EST programmes, in cereal species such as barley (Ozturk *et al*., 2002), and rice (Kawasaki *et al*., 2001) after generating non-redundant unigene sets. Transcript profiling based on micro/macro-arrays was carried out in cereal crops (Ozturk *et al*., 2002; Sreenivasulu *et al*., 2004b) as well as in Arabidopsis (Seki *et al*, 2002a, 2002b) to analyse gene expression in response to a variety of stresses. Most of the recent emphasis has been made on dissecting the mechanisms of dehydration responses in vegetative tissues triggering gene expression associated with desiccation tolerance in an ABA-dependent manner via ABA-responsive element binding factors (ABF), MYC and MYB transcription factors and in an ABA-independent manner via drought-responsive element binding factors (DREB). As an outcome of transcriptome studies it is apparent that ABA is not only involved in drought-specific responses but also there is a cross-talk in cold and salinity stress responses (Seki *et al*., 2002a,b).. The transcriptome analysis and prediction of *cis* elements in tightly co-expressed gene set indicates that embryo-specific gene expression patterns show peak of expression during late maturation delineate a regulatory network for the acquisition of desiccation tolerance, which are controlled by ABA mediated signal transduction via ABF and in an ABA independent manner via DREB 2A transcription factor. Further, the same coupling regulatory factors ABF and DREB triggers oleosin and lipid biosynthesis genes which are co expressed with desiccation-related LEA genes. Such comparative studies confirm the participation of well known regulators described in vegetative tissues during dehydration responses also in the natural process of desiccation tolerance in seeds.

In model legumes, extensive sequencing highlighted around 2,000 Transcription Factors per genome, of which less than 1% have been genetically characterized (Udvardi *et al*., 2007). Transcriptome data covering natural acquisition of desiccation tolerance during embryo maturation and transcriptional changes occurring in 3 mm long radicles of *Medicago truncatula* seeds leading to

drought tolerance were compared (Buitink *et al*., 2006). Mt16K+ microarrays covering 16,086 tentative consensus sequences derived mainly from 164,000 *M. truncatula* ESTs collected in the TIGR *M. truncatula* Gene Index 5 were used to monitor changes in the transcriptome of desiccation-sensitive radicles of *M. truncatula* seeds. In this work, more than 1,300 genes were differentially expressed during embryo desiccation and several regulatory genes (including TFs) were up-regulated during maturation. The protective mechanisms had clear overlap of ABA-dependent and ABA independent regulatory pathways involved in both drought and desiccation tolerance. Transcriptome response to dehydration, salinity and ABA has been monitored in sorghum seedlings and identified approximately 22 transcription factors (Buchanan *et al*., 2005). These regulators include ABF from bZIP factors, DREB from AP2/EREBP family, HD-ZIP and MYB factors, which are also known to be stress-responsive in other model species such as Arabidopsis and rice. Also, there is a greater need to verify the roles that these transcription factors play in the networks for better designing plants that can tolerate a variety of environmental stresses. Sakuma *et al*., (2006) addressed this interesting issue by performing transcriptome studies in overexpression lines of DREB2 and DREB1 transgenic plants and identified only 8 genes in common. The remaining 14 genes are probable targets of DREB2a which consists of at least 9 LEA members thought to confer dehydration tolerance. Further, by performing promoter analysis and gel mobility shift assay of the DREB2a and DREB1a up-regulated genes authors concluded that the DREB2a and DREB1a proteins have different binding specificities, therefore genes downstream of DREB2a and DREB1a trigger different set of genes conferring drought and freezing tolerance, respectively. Recently, transcription profiling revealed 912 genes differentially expressed during salt acclimation in *Lotus japonicas* (Sanchez *et al*., 2008). These transcriptional changes were presumably coordinated by specific TFs modulated during this process such as AP2/ERF and MYB (24 and 20% of the total number of TFs, respectively).

CONCLUSION AND FUTURE PERSPECTIVE

After the discovery of genomic tools such as molecular markers, genetic maps, etc., plant breeding has been facilitated greatly and improved genotypes/varieties with enhanced resistance/tolerance to biotic/abiotic stresses have been developed in several crop species. To increase the genomic resources in commercially important crops like rice wheat and many other grain legumes and oilseed crops transcriptome sequencing to generate ESTs is a method of choice. ESTs, which are generated by large-scale single pass sequencing of randomly picked cDNA clones, have been cheap and valuable resource for efficient and rapid identification of novel genes and development of molecular markers. ESTs have also been employed in bioinformatic analyses to discover the genes that are differentially expressed in various tissues, cell types, or developmental stages of the same or different genotypes. Hence, the availability of large amounts of data in terms of ESTs will provide an opportunity for the discovery of novel genes responsible for tolerance against the yield affecting biotic and abiotic stresses in crop plants.

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