Advances in Omics for Enhancing Abiotic Stress Tolerance in Millets

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Millets are small-seeded annual cereal crops which are mostly grown in marginal soils of arid and semi-arid regions, and consequently have to face several abiotic constraints that lead to reduction in the yield. Advanced biotechnological applications, particularly 'omics' approaches could serve as the most immediate and prospective strategies for improving abiotic stress tolerance in millet crops. Omics approaches lead to understand the mechanism of stress response and tolerance at molecular level through insights into gene, protein or metabolite profile and their phenotypic effects. Among millet crops, foxtail millet has been more intensively studied and characterized under abiotic stress conditions owing to its small genome size and diploidy. Genome-wide investigation and expression profiling studies have been extensively carried out in foxtail millet. However proteomic and metabolomic studies in response to abiotic stress in millets are still very limited. Some of the major quantitative trait loci (QTLs) controlling abiotic stress tolerance in millets have also been identified and mapped as for instance a major QTL for terminal drought tolerance in pearl millet and a major QTL for dehydration tolerance in foxtail millet. Gene manipulation for enhanced abiotic stress tolerance involving regulatory genes have proved to be more useful than using single or multiple stress responsive genes. Consequently, the recent advances in 'omics' technologies and accessibility to the genome sequences extend huge potential to enhance stress tolerance of millets. This review thus attempts to summarize various omics strategies and their applications in abiotic stress research in important millet crops namely foxtail millet, pearl millet and finger millet.

Key Words : Abiotic Stress; Genomics; Millets; Metabolomics; Omics; Phenomics; Proteomics; Transcriptomics

Introduction

Plants being sessile in nature are forced to thrive in stressful conditions originating either from physical environment (abiotic stress) or due to interactions with other living organisms (biotic stress). Abiotic stresses such as drought, salinity, water logging, extreme temperatures and heavy metals pose serious constraint to plant growth and development leading to yield reduction by more than 50% for major crop plants (Bray *et al.*, 2000). Agricultural land that fall under non-stress category accounts for only 10% of the total arable land across the globe implying that crops raised on rest cultivable lands are subjected to one or more abiotic stresses (Dita *et al.*, 2006). Moreover, crops which suffer abiotic stresses are usually more prone to weeds, insects, and pathogens adding up the losses considerably (Reddy *et al.*, 2004). Response and adaptation in order to survive and reproduce under such stress conditions is a complex phenomenon and is by and large synchronized and adjusted by regulating crop's physiological, cellular and molecular activities (Chinnusamy *et al.*, 2004; Ahuja *et al.*, 2010). Hence understanding the mechanisms of plant stress tolerance and adaptation have long been the focal point of extensive and rigorous

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studies by researchers. With the progression of omics technologies and functional characterization of individual genes, it has become evident that the environmental acclimatization which is crucial for plant survival is under strong regulation (Lopez *et al.*, 2008; Muthamilarasan and Prasad, 2014; Kissoudis *et al.*, 2014). It has been established that several components are involved in stress regulatory networks and may function synergistically or antagonistically to each other, thus promoting or compromising plant resistance to various abiotic stresses occurring separately or concurrently (Glazebrook, 2005; Kissoudis *et al.*, 2014). Significant progress has been accomplished towards deciphering stress tolerance mechanisms not only in model crop like *Arabidopsis* but also in other crops like garden and woody species, cereals and millets (Bokhari *et al.*, 2007; Alvarez *et al.*, 2008; Caruso *et al.*, 2009; Degenkolbe *et al.*, 2009; Shulaev *et al.*, 2008; Watkinson *et al.*, 2008; Zellar *et al.*, 2009; Ginzberg *et al.*, 2009; Dwivedi *et al.*, 2012).

'Millets' is a collective term used to refer a diverse group of small-seeded annual C_4 Panicoid grasses such as foxtail millet (*Setaria italica*), pearl millet (*Pennisetum glaucum*), finger millet (*Elusine coracana*), proso millet (*Panicum miliaceum*) etc. that are cultivated as food and fodder crops, primarily grown on poor and marginal lands in dry areas of temperate, subtropical and tropical regions across the globe (Dwivedi *et al.*, 2012; Lata *et al.*, 2013). Millets characteristically adapt to unfavorable ecological conditions including abiotic and biotic stresses, demand least inputs and have remarkable nutritional properties. Nutritional quality of millet grains is usually equivalent or superior to other cereals with high amounts of carbohydrates, proteins, minerals, vitamins and essential amino acids (Dwivedi *et al.*, 2012). Though millets rank sixth in world grain production after rice, wheat, maize, barley and sorghum, they are usually considered as a subsistence product and more often than not looked upon as a poor man's crop (Dwivedi *et al.*, 2012; Lata *et al.*, 2013). Noteworthy, India ranks first in global millet production with an annual production of ~11 million tonnes in 2013 (FAOSTAT, 2014; http://faostat.fao.org/site/567/Desktop Default.aspx?PageID=567#ancor). Millets epitomize important genomic resources for agriculture as well

as nutritional security of poor and marginal farmers that dwell in arid and semi-arid regions across the globe. Despite their significance, millets have largely been an under researched crop commodity as they are considered minor cereal crops of only regional importance, and hence little attention has been given for developing their genetic and genomic resources for use in crop improvement as compared to other staple cereals. Since millets are grown in low-input, rain-fed agriculture system, they tend to suffer from drought spells due to scarce, untimely and irregular rainfall that ultimately becomes major constraint for crop yield. Besides drought, soil salinity and high temperature are another major abiotic stress factors that severely affect crop growth and productivity. Therefore, it is important to improve abiotic stress tolerance in crop plants to ensure enhanced yield stability. Though conventional plant breeding along with good crop management practices have addressed several agronomic constraints that reduce crop yield or nutritional quality, there may be cases where the existing genetic resources do not possess the requisite traits. Yield losses due to abiotic constraints such as drought are highly variable in nature owing to stress timing, intensity and duration. Further breeding for drought tolerance through conventional approaches becomes even more difficult due to the occurrence of some location-specific stress factors such as high temperature or irradiance. Therefore advanced plant breeding practices supported effectively by cutting-edge genomics and genetic transformation technologies could lead to simpler and successful gene-based approaches for enhancing abiotic stress tolerance of millet crops and thereby contributing to sustainable agriculture of dry regions. Among millets however, foxtail millet, pearl millet, and to lesser extent finger millet have lately started gaining some importance among the research community wherein 'omics' have played an important role apart from conventional plant breeding.

The Concept of Omics and Abiotic Stress Tolerance in Millets

Successful application of biotechnological approaches to overcome abiotic constraints entails a sound knowledge of both the target crop species and the

underlying stress tolerance mechanisms that encompasses measurements from the whole plant to molecular level. Plant stress response is basically influenced by the type, duration, and severity of stress (Bray, 1997). Given that responses are governed by plant genome, recent efforts have been focused on decoding molecular response of the plants to abiotic stresses. Until recently, model plants such as *Arabidopsis* and rice were the focal point of research pertaining to studies on plant stress responses, while orphan crops like millets lagged far behind. Since large genome size and polyploidy hindered the basic goal to unravel the abiotic stress tolerance mechanisms in these naturally stress tolerant crops, recent progress in this area has been significantly enhanced by the development of foxtail millet as a model system to investigate evolution, physiology and the genetics of stress tolerance in millets. Foxtail millet has been the obvious choice for model millet crop owing to its small genome size, diploidy (2n=2x=18), self pollination, short generation time and availability of efficient transformation platform (Lata *et al.*, 2013). Powerful genetic and genomic tools such as isolation of expressed sequence tags (ESTs), genome sequencing, genetic and physical mapping, comparative mapping etc. have been thus established for the molecular genetic studies in millet crops (Dwivedi *et al.*, 2012; Lata *et al.*, 2013; Muthamilarasan and Prasad, 2014). The foxtail millet genome sequence and the high degree of synteny between foxtail millet and switch grass (*Panicum virgatum*), a bioenergy crop have a potential to facilitate genetic and genomic studies in related Panicoid crops (Lata *et al.*, 2013; Lata and Prasad, 2013a).

Though sequence information is indispensable and essential starting point for molecular biology, it is alone not enough to resolve queries pertaining to stress responsive gene function, regulatory networks, and the biochemical pathways involved. Therefore, it becomes necessary to include more comprehensive approaches such as quantitative and qualitative analyses of gene expression products at the transcriptomic, proteomic, and metabolomic levels along with bioinformatics and systems biology approaches which will further allow a more specific use of marker assisted selection (MAS) and transgenic

technologies (Dita *et al.*, 2006). 'Omics' is generally used to refer all biotechnological applications that requires knowledge of stress response at molecular level including genomics, functional genomics, genetic engineering, gene expression, protein or metabolite profile(s) and their overall phenotypic effects in response to environmental perturbations which are generally accompanied by significant alterations in the plant transcriptome, proteome and metabolome (Ahuja *et al.*, 2010; Fig. 1). Recently 'omic' approaches are being efficiently utilized to understand the molecular and genetic basis of abiotic stress perception, response and tolerance in millet crops. Transcriptome and expression profiling studies have been routinely carried out in various millet crops to identify candidate genes and regulatory networks involved in abiotic stress responses. Until now noteworthy progress has been achieved in abiotic stress research of major millets including foxtail millet, pearl millet, and finger millet as elaborated in the subsequent sections.
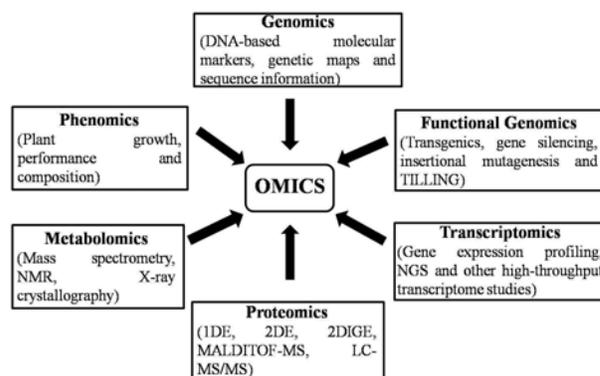

Fig. 1: A schematic representation of different Omics strategies

Genomics

Molecular markers, genetic maps and sequence information are some of the basic genomic resources required for genetic studies and molecular breeding approaches. Therefore genomics as a whole involves development of molecular markers and manipulation of quantitative trait loci (QTLs) through MAS for development of improved cultivars. These genomic resources facilitate breeding strategies for crop improvement programs by reducing the time- and labor-intensive direct screening of germplasm grown

under field and green house conditions. Genetic maps and molecular markers also help in comparative mapping and synteny studies in crop plants, for example one of the recent studies have shown the synteny between pearl millet and foxtail millet chromosomes along with those of other grasses (Rajaram *et al.*, 2013; Fig. 2). However discovery of DNA markers and construction of genetic linkage maps in millets have lagged behind other cereal crops such as rice, wheat and maize (Dwivedi *et al.*, 2012). Among millets, mainly foxtail millet, pearl millet, finger millet, job's tear (*Coix lacrymajobi*) and tef (*Eragrotis tef*) have been investigated for development of genetic and genomic resources (Dwivedi *et al.*, 2012).

Foxtail Millet

The availability of foxtail millet genome sequence together with genetic and physical maps provide valuable resources for powerful and efficient identification and characterization of genes and QTLs for various agronomic traits, thus facilitating marker-

assisted breeding (MAB) and crop improvement. The first foxtail millet restriction fragment length polymorphism (RFLP)-based map consisting of 160 loci was constructed in an intervarietal cross (Wang *et al.*, 1998) which was further proven to be useful in creating comparative genetic maps of foxtail millet and rice (Devos *et al.*, 1998). Since then several groups reported on development of various genomic or EST based simple sequence repeat (SSR) markers to be used in diverse genotyping applications, phylogenetic and transferability studies in foxtail millet and other grass species (Jia *et al.*, 2007; Jia *et al.*, 2009; Heng *et al.*, 2011; Gupta *et al.*, 2012; Gupta *et al.*, 2013). Considering the importance of intron length polymorphic (ILP) markers as genomic resources, Gupta *et al.* (2011) and Muthamilarasan *et al.* (2014a) developed 98 and 5123 ILP markers respectively in foxtail millet. The ILP markers from both studies showed to be useful in cross-species transferability, germplasm characterization, comparative mapping and genomic relationships studies between millets and non-millets.
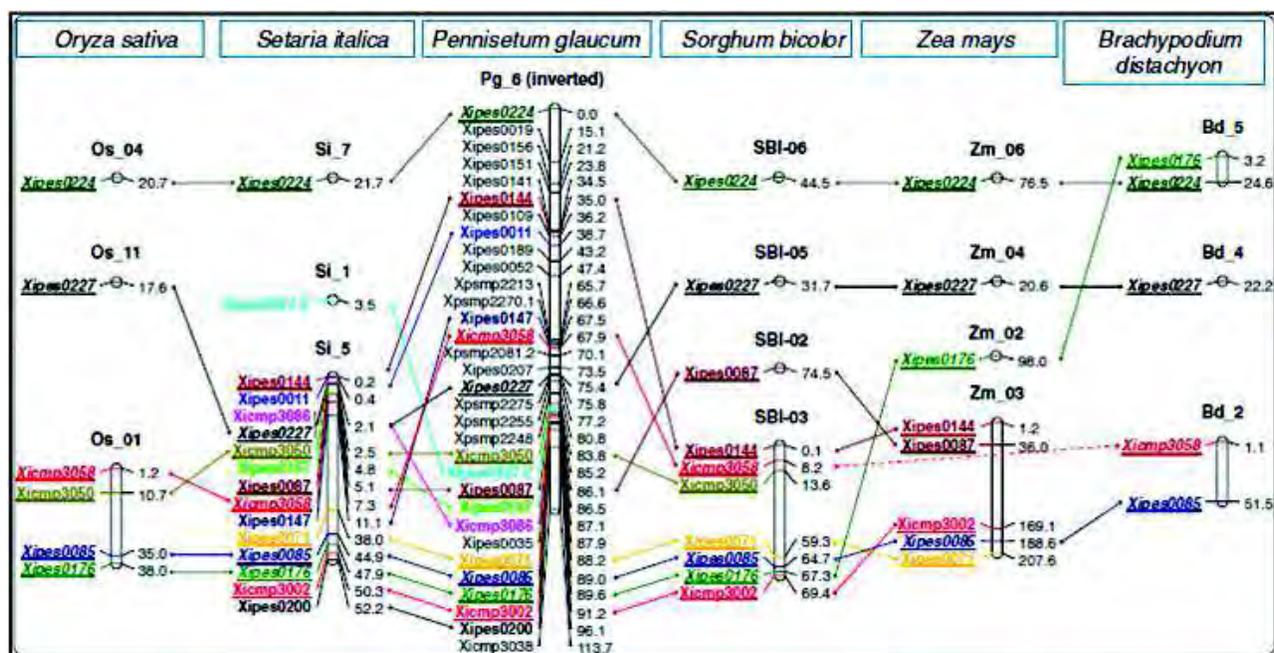

Fig. 2: A representation of synteny between pearl millet linkage group 6 (inverted) with five sequenced grass genomes including foxtail millet. Pearl millet has 7 (Pg_1 to Pg_7), foxtail millet has 9 (Si_1 to Si_9), rice has 12 (Os_1 to Os_12), sorghum has 10 (Sb_1 to Sb_10), maize has 10 (Zm_1 to Zm_10) and *Brachypodium* has 5 (Bd_1 to Bd_5) linkage groups. EST-SSRs mapped on linkage group 6 of pearl millet. Linkage distances (in cM for pearl millet) or physical map positions (in Mb for other grasses) are given on the right side of each bar and the marker names are given on the left side of each bar. (Adapted from Rajaram *et al.*, 2013)

With the availability of *Setaria* genome sequence it has become possible for large-scale development of foxtail millet genomic resources (Bennetzen *et al.*, 2012; Zhang *et al.*, 2012). A foxtail millet landrace 'Shi-Li-Xiang' was resequenced to identify single nucleotide polymorphisms (SNPs), InDels, and structural variations (SVs) as compared to two foxtail millet reference genome sequences in order to expedite the identification of important candidate genes to be used for molecular breeding (Bai *et al.*, 2013). Xu *et al.* (2013) developed a total of 9,576 and 7,056 SSR markers from 42,754 transcripts generated from referenced based assembly using foxtail millet genome and 60,751 transcripts from *de novo* assembly, respectively. Several other researchers also reported on identification of several microsatellite repeat motifs (Pandey *et al.*, 2013; Zhang *et al.,* 2014), eSSRs (Kumari *et al.*, 2013), and transposable elements (TE)-based markers (Yadav *et al.*, 2014a) using *Setaria* genome sequence data. In another interesting study, 176 microRNA (miRNA)-based molecular markers have been developed (Yadav *et al.*, 2014b) from a set of 335 mature miRNAs (Khan *et al.*, 2014). SNPs are single base substitution, insertion or deletion commonly found in a population and have recently been developed into powerful molecular markers. Lata *et al.* (2011a) identified a synonymous SNP associated with dehydration tolerance in a novel DREB2-like gene and developed an allele-specific marker (ASM) for the same. The ASM was validated in a core set of more than 100 foxtail millet accessions and the regression of lipid peroxidation (LP) and relative water content (RWC) on the ASM suggested that the *SiDREB2*-associated trait contributed to ~ 27% and ~20% of the total variation, respectively demonstrating the importance of this QTL for dehydration tolerance in foxtail millet (Lata and Prasad, 2012; Lata and Prasad, 2013b). Further, a total of 2,584,083 SNPs were identified from 916 diverse foxtail millet accessions, out of which 845,787 SNPs (minor allele frequency >0.05) were used to construct a foxtail millet haplotype map (Jia *et al.*, 2013).

These marker resources are efficiently utilized time to time in genomics-assisted breeding. There have been several reports where molecular markers such as amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR) and SSRs have been used in assessing genetic relationships, studying domestication-related geographical structure and phylogenetic relationships between foxtail millet and wild relatives (Le Thierry d'Ennequin *et al.*, 2000; Li *et al.*, 2012; Wang *et al.*, 2012), evaluating genetic diversity, population structure and linkage disequilibrium (LD) in various foxtail accessions (Li *et al.*, 2012; Vetriventhan *et al.*, 2014) as well as for examining important agronomic traits (Mauro-Herrera *et al.*, 2013; Gupta *et al.*, 2014) which once validated could be useful for identification of genes/QTLs linked with important traits and eventually in MAB of foxtail millet and related crops (Muthamilarasan and Prasad, 2014). To assist this, comprehensive online resources such as marker database (FmMDb; http://www.nipgr.res.in/foxtail.html) (Suresh *et al.*, 2013) and transcription factor database (FmTFDb; http://59.163.192.91/FmTFDb/) (Bonthala *et al.*, 2014) have been constructed to provide the researchers and breeders unrestricted access to the developed genetic and genomic resources.

Pearl Millet

Pearl millet is one of the most hardy cereal crops grown for grain, forage and stover in the arid and semi arid tropical regions of Asia and Africa. It is an annual, small seeded, highly cross pollinated, C_4 Panicoid cereal crop (2n=2x=14) with a very large genome size (~2400Mb) and short life cycle (Vadez *et al.*, 2012). It has substantial stock of genetic and genomic resources with various DNA-based molecular markers including RFLP (Liu *et al.*, 1994); sequence-tagged sites (STS; Devos *et al.*, 1995); AFLP (Allouis *et al.*, 2001); SSRs (Qi *et al.*, 2004); diversity arrays technology (DArTs; Supriya *et al.*, 2011); SNPs and conserved intron spanning primer (CISP) markers (Sehgal *et al.*, 2012); mapping populations, and DNA-marker based linkage maps (Vadez *et al.*, 2012; Sehgal *et al.*, 2012). The genetic maps so developed have been demonstrated to be valuable not only for detection and breeding of promising QTLs for various traits (Jones *et al.*, 1995; Morgan *et al.*, 1998; Jones *et al.*, 2002; Nepolean *et al.*, 2006; Gulia *et al.*, 2007; Bidinger *et al.*, 2007)

including terminal drought tolerance (Yadav *et al.*, 2002; Yadav *et al.*, 2004), components of drought adaptation (Kholová *et al.*, 2012) and grain and stover yield (Yadav *et al.*, 2003) but also for better understanding of complex relationships between pearl millet and other cereal crops (Devos *et al.*, 2000).

A major QTL for terminal drought tolerance in pearl millet has been identified and mapped on linkage group 2 (LG 2) using segregating populations derived from two independent crosses between H 77/833-2 and PRLT 2/89-33, and ICMB 841 and 863B (Serraj *et al.*, 2005; Bidinger *et al.*, 2005; Bidinger *et al.*, 2007). This QTL on LG 2 has been considered a major target for MAS for improving grain yield and grain stability across variable terminal stress conditions in pearl millet (Yadav *et al.*, 2011). This DT-QTL has also been found to confer high leaf abscisic acid (ABA) and limiting transpiration rates at high vapor pressure deficits supporting the hypothesis of water saving mechanisms under well-watered conditions in drought tolerant pearl millet lines (Kholová *et al.*, 2010). A major grain yield (GRYLD) QTL was also identified on LG 2 which also co-mapped with QTLs for harvest index (HI), grain number and grain mass under severe terminal stress (Bidinger, 2007). Other than these, several other QTLs associated with drought tolerance of grain yield in late stress environments were identified on various other linkage groups such as LG 3, 4, 5 and 6 (Yadav *et al.*, 2004; Bidinger *et al.*, 2007). Performance of LG 2 major QTL for terminal drought tolerance was also assessed under salt stress and interestingly it was found that this DT-QTL enhanced growth and productivity traits under saline and alkaline conditions by limiting Na^+ accumulation in pearl millet leaves (Sharma *et al.*, 2011). Another study established that the drought tolerant parent (PRLT 2/89-33) and two QTL-near isogenic lines (NILs) recorded higher yield under salinity stress at post-flowering growth stages as compared to drought sensitive parent (Sharma *et al.*, 2014).

Finger Millet

Finger millet is another important climate resilient nutri-millet which has a rich resource of superior genes

and alleles (Solanke and Mithra, 2013). It is an allotetraploid (2n=2x=36) with genome designated as AABB. Breeding efforts in this crop have been limited owing to its highly self-pollinating nature and small flowers. Further, germplasm pool of this crop also remains largely uncharacterized with a very few reports in this regard to date based on morphological, nutritional and other quantitative traits (Prasada Rao and de Wet, 1997; Vadivoo *et al.*, 1998; Upadhyaya *et al.*, 2006). Dida *et al.* (2008) have reported wider level of genetic diversity among several domesticated finger millet cultivars through RAPD and microsatellite approach. Generation of a RFLP, AFLP and SSR marker-based genetic linkage map of its A and B genomes was the first major breakthrough in the area of finger millet genomics (Dida *et al.*, 2007). A comparative analysis between finger millet and rice was carried out that revealed high level of conserved colinearity between gene orders. The study also led to deduce the supposed ancestral chromosome configurations in grass species. There have been quite a few reports on genomics studies for biotic stress resistance in finger millet (Panwar *et al.*, 2010; Panwar *et al.*, 2011; Babu *et al.*, 2014), the same pertaining to abiotic stress research in finger millet is not available, and thus emphasizing the need of focused research in this regard.

Details of DNA-based molecular markers and genetic maps reported in foxtail millet, pearl millet and finger millet is given in Table 1.

Functional Genomics

In the post genomics era, comprehensive analysis using functional genomics technologies such as overexpression, gene silencing, insertional mutagenesis and targeted induced local lesion in genome (TILLING) have played an important role in improving our knowledge on complex regulatory networks associated with stress response, adaptation and tolerance in plants. Different omics technologies are also providing huge data sets which can be exploited to identify important candidate genes to be used in crop improvement programs through MAS or transgenic technology in millets. With the advancement of genome sequencing projects and proteomics, the

Table 1. List of molecular markers and genetic linkage maps reported in foxtail millet, pearl millet and finger millet

DNA markers	Genetic maps	References
Foxtail millet		
5123 Intron length polymorphic (ILP) markers	4049 ILP markers physically mapped on 9 foxtail millet chromosomes	Muthamilarasan *et al.*, 2014a
5020 highly repetitive microsatellites motifs	A physical map of 733 highly polymorphic SSR markers developed	Zhang *et al.*, 2014
20,278 transposable element-based markers		Yadav *et al.*, 2014a
176 miRNA-based markers		Yadav *et al.*, 2014b
9,576 and 7,056 SSR markers		Xu *et al.*, 2013
28, 342 microsatellites repeat motifs	A high density physical map based on 28,342 microsatellites repeat motifs	Pandey *et al.*, 2013
534 eSSRs	A physical map based on 327 eSSR markers	Kumari *et al.*, 2013
64 genomic SSR markers		Gupta *et al.*, 2013
147 genomic microsatellite (SSRs) markers		Gupta *et al.*, 2012
98 ILP markers		Gupta *et al.*, 2011
One Allele-specific marker for SiDREB2 gene		Lata *et al.*, 2011a
~1000 SNPs by sequencing pools of RILs (S. italica acc. B100 x S. virdis acc. A10)	A genetic map with ~1000 SNPs evenly mapped to all 9 chromosomes	http://www.plantbio.uga.edu/media/2010_grad_symposium(1).pdf
12 EST-derived SSRs		Nnaemeka, 2009
100 polymorphic SSRs from two genomic DNA libraries	A genetic map with 81 SSRs and 20 RFLP markers mapped onto 9 chromosomes covering 1654 cM and marker density of 16.4 cM	Jia *et al.*, 2009
160 RFLP markers	160 RFLP loci mapped onto 9 LGs spanning 964 cM	Wang *et al.*, 1998
Pearl millet		
99 polymorphic EST-SSR markers	116 EST-SSRs, 53 genomic SSRs and 2 STS markers mapped on one or more of the four F7 RIPs	Rajaram *et al.*, 2013
75 gene-based markers (SNP and CISP)		Sehgal *et al.*, 2012
574 polymorphic DArT markers	A genetic linkage map using 258 DArTs and 63 SSRs spanning 1148cM	Supriya *et al.*, 2011
58 EST- SSR primer pairs	17 EST-SSR markers mapped to 5 of the 7 pearl millet LGs	Senthilvel *et al.*, 2008
19 EST-derived SSR primer pairs		Yadav *et al.*, 2007
16 EST-derived polymorphic SSRs		Mariac *et al.*, 2006
SSCP–SNP primer pairs		Bertin *et al.*, 2005
44 SSRs derived from genomic library	A genetic map using 353 RFLP and 65 SSRs	Qi *et al.*, 2004
	A genetic map with 61 RFLP and 30 SSR loci spanning 476 cM	Yadav *et al.*, 2004
18 SSRs derived from genomic library		Budak *et al.*, 2003;
25 SSR markers from BAC library		Qi *et al.*, 2001
	A genetic map with 38 RFLP markers covering 280 cM	Jones *et al.*, 1995
	181 RFLP loci mapped to 7 LGs spanning 303 cM	Liu *et al.*, 1994
Finger millet		
36 EST-SSRs and 20 anchored-SSRs		Nirgude *et al.*, 2014
3 EST-derived SSRs		Nnaemeka, 2009
31EST-derived SSRs		Arya *et al.*, 2009
82 SSR markers	A genetic linkage map with 131 markers mapped to 16 LGs spanning 721 cM on the A genome and 196 markers mapped to 9 LGs covering 787 cM on the B Genome	Dida *et al.*, 2007
	332 loci from 266 primer pairs mapped to 26 LGs Highly conserved gene orders between rice and finger millet	Srinivasachary *et al.*, 2007

challenge remains to functionally validate hundreds and thousands of identified genes or proteins where reverse genetics strategies will be crucial. A powerful tool for determining function of a gene is to overexpress it in same or heterologous system under the control of a constitutive or stress-inducible promoter. While another important strategy to determine function of a gene is to suppress its expression or knock it out. Significant work regarding this has been initiated in millets.

Transgenomics

Transgenomics or transgenic technology is a targeted gene-based functional genomics tool which provides valuable information about regulatory mechanisms underlying stress tolerance in plants. Hence in recent years transgenic technology has widely been used for genetic improvement of many crop plants including major cereals. However, transgenomics in millets is still in nascent stage despite their economic and nutritional importance, though there are several reports where candidate genes from millets have been functionally validated in *Arabidopsis* or tobacco (Agarwal *et al.*, 2007; Ramegowda *et al.*, 2012; Yue, 2014). Most of the above candidate genes from millet crops are transcription factors (TFs) as they can regulate the expression of downstream stress responsive genes and thus play important role in imparting stress tolerance. Of note, several candidate TFs including AP2/ERFs, NAC, C_2H_2 zinc finger and MYB were analyzed in foxtail millet to identify suitable candidate genes which can be used for further characterization using transgene-based approaches towards engineering stress resistance (Lata *et al.*, 2014; Puranik *et al.*, 2013; Muthamilarasan *et al.*, 2014b; Muthamilarasan *et al.*, 2014c). In addition to TFs, several stress-responsive genes such as WD40, Dicer-like (DCL), Argonaute (AGO) and RNA-dependent RNA polymerases (RDR) have been studied in foxtail millet to identify potential candidate genes for downstream characterization (Mishra *et al.*, 2014; Yadav *et al.*, 2014c). Despite these advances, development of protocols for generating transgenic lines in millets is still in its early stage. An efficient *in vitro* regeneration protocol particularly through somatic embryogenesis is crucial for establishment

of successful transformation and recovery in any crop plant (Veluthambi *et al.*, 2003). There have been quite a few reports on somatic embryogenesis and plant regeneration protocols in foxtail millet, pearl millet and finger millet (Kothari *et al.*, 2005).

Foxtail Millet

Foxtail millet is an important target for genetic transformation among millet crops owing to its close similarity to potential bioenergy grasses. Particle bombardment and *Agrobacterium*-mediated genetic transformation are important transgenomics approaches, however, poor transformation efficiency makes particle bombardment a least preferred choice. Liu *et al.* (2009) reported transformation of *SiPf40* through particle bombardment in foxtail millet where pROKf40s, pROKf40an and pROKf40i plasmids were used. *Agrobacterium*-mediated transformation is more widely used but there has been only one report in foxtail millet for improved abiotic stress tolerance. Li *et al.* (2014) reported transformation of *SiARDP*, a target gene of *SiAREB* in foxtail millet and *Arabidopsis* and found it to play crucial role in drought stress tolerance.

Pearl Millet

Biolistic method of gene delivery has mostly been used for pearl millet transformation (Ceasar and Ignacimuthu, 2009). The first report on pearl millet transformation through particle bombardment was by Taylor *et al.* (1991) where plasmid pMON8678 was used for transformation. Later several groups reported on biolistic transformation of this crop with different target tissues and transgenics were raised against fungal pathogens (Ceasar and Ignacimuthu, 2009). However no report on genetically engineered improved abiotic stress tolerant pearl millet has been reported till date. Only recently *Agrobacterium*-mediated transformation using shoot apices have been reported in pearl millet (Jha *et al.*, 2011; Ignacimuthu and Kannan, 2013).

Finger Millet

The genetic improvement of finger millet through transgenic technology has been limited despite its

nutritional significance as well as the fact that improved grain yield conferring resistance to other stresses is a priority in this crop. The two earlier reports on finger millet transformation were through biolistic method (Gupta *et al.*, 2001; Latha *et al.*, 2005). *Agrobacterium*-mediated transformation of finger millet using shoot explants was established by Ceasar and Ignacimuthu (2011). There has been only one report on generation of transgenic finger millet with improved multiple stress tolerance including drought, salinity and oxidative stress (Hema *et al.*, 2014). In this study the tolerance in finger millet was achieved through the stable expression of *mannitol-1-phosphate dehydrogenase* (*mtlD*) gene from bacteria.

Gene Silencing

Gene silencing is an important reverse genetics approach used to determine function of a known gene that leads to altered phenotypes. Insertional mutagenesis (T-DNA insertions or transposable elements insertion) is one of the gene silencing methods which is used to knock out expression of a target mRNA but the process suffers certain limitations as in the case of multigene family members, and thus the process requires considerable analyses for selection of desired genes. Also it has not been possible to achieve targeted gene replacement via homologous recombination in higher plants to date. Recently virus-induced gene silencing (VIGS) through dsRNA has emerged as an important functional genomics tool which is relatively easy and rapid (Baulcombe, 2004). VIGS suppresses target plant gene expression through virus vectors that harbors a target region of the host gene. In general, it does not produce knockout mutants nor does it generate stable RNA interference (RNAi) and can also be carried out in species which are not easy to transform (Scofield and Nelson, 2009). Though several vectors are available for supporting VIGS in crop plants, they have yet not been extensively utilized in millets. Foxtail millet due to its close proximity to several bioenergy grasses has been adopted as a high-throughput platform for VIGS to model gene inhibition outcomes (Doust *et al.*, 2009). Ramanna *et al.* (2011) have developed VIGS protocol for functional characterization of cell wall related genes in foxtail millet where in Brome mosaic virus was used as the vector (http://cdm16483.contentdm.oclc.org/cdm/ref/collection/p15481coll2/id/3763). A VIGS system has also been developed in pearl millet (Turina *et al.*, 1998; van Nugteren *et al.*, 2007). However until now there has been no report on utilization of VIGS for enhancing abiotic stress tolerance in millet crops.

Transcriptomics

'Transcriptome' refers to the total population of messenger RNAs (mRNAs) in a cell or tissue or organism and the investigation thereof is termed as 'transcriptomics'. Subsequently genome-wide expression profiling studies serve as powerful tools for identifying candidate genes involved in various biological processes and stress regulatory networks. Plants exposed to drought, salinity or temperature extremes or ABA generally undergo cellular dehydration and hence almost half of the genes expressed in all these stresses are common (Reddy *et al.*, 2012). Normally, the abiotic stress responsive genes can broadly be categorized into two classes on the basis of their response in terms of duration, namely early- and late-responsive where early responsive genes respond immediately to stress within a few seconds or minutes, the late responsive ones may take hours, days or even weeks for their expression (Ramanjulu and Bartels, 2002). Hence it is envisaged that the early responsive genes such as protein kinases and TFs are implicated in providing initial protection and regulating gene expression by being themselves directly involved in signal perception, amplification and transduction. While the late responsive genes encode for proteins such as late embryogenesis abundant (LEA) proteins, heat shock proteins (HSPs), reactive oxygen species (ROS) scavengers etc. and are speculated to help in stress adaptation (Bray, 1997; Ramanjulu and Bartels, 2002). Also it has been reported that 11% of stress-inducible genes are essentially potential TFs supporting the significance of gene regulation in stress adaptation and tolerance (Seki *et al.*, 2002).

The transcriptomic approach *per se* allows comprehensive analysis and quantification of changes induced by abiotic stresses at whole organism level.

Among monocots, rice has been extensively investigated for gene expression studies and the results so obtained could also be used for comparative genomics in millets. However, the rice model is likely to be quite diverse from millets in terms of stress response, physiology, architecture and metabolism since physiological processes between C_3 and C_4 crops are essentially different. Therefore developing a C_4 grass model system has become increasingly pertinent in the present scenario. Significant progress in millets research at genetic and genomic levels has been made using model C_4 crop foxtail millet (Lata *et al.*, 2013) through genome sequencing, macro- and microarray analysis and other high throughput techniques (Lata and Prasad, 2012; Lata *et al.*, 2013). *Setaria* genome sequence is available for public use at http://www.phytozome.net/foxtailmillet.php. The next generation sequencing platforms have revolutionized scientific research as they allow deciphering sequences of cell transcripts within hours. Hence this approach has the ability to circumvent the problems posed by extremely large genomes such as those of millets.

Foxtail Millet

Foxtail millet is a naturally drought stress tolerant crop having higher water use efficiency (WUE) as compared to maize, wheat and sorghum (Zhang *et al.*, 2007; Lata *et al.*, 2013). Recently, the whole transcriptome of foxtail millet was analyzed through deep sequencing that led to the identification of drought-responsive 2,824 genes with differential expression patterns, out of which 48.23% and 51.77% were up-, down-regulated, respectively (Qi *et al.*, 2013). LEA proteins, dehydrins, HSPs, aquaporins and phosphatase 2C were the most abundant up-regulated proteins suggesting their possible role in imparting drought tolerance to foxtail millet. Transcript profiling of foxtail millet exposed to drought stress was also carried out by Yin *et al.* (2014). In this study an F-box protein was found to be involved drought and ABA response. *Setaria* genome sequencing project also revealed the presence of 586 'response to water' genes which are predicted to play significant roles in stress response and adaptation (Zhang *et al.*, 2012). Earlier Zhang *et al.* (2007) identified several drought-

induced differentially expressed genes in foxtail millet using suppression subtractive hybridization and cDNA microarray. The expression analysis of these genes indicated that distinct sets of genes are activated in roots and shoots of foxtail millet on drought stress treatment and interestingly majority of them participate in protein degradation pathway. In a similar study, 86 up-regulated genes were identified at 0.5h and 6h of polyethylene-induced dehydration stress in tolerant foxtail millet cultivar 'Prasad' (Lata *et al.*, 2010). In order to understand the salinity tolerance mechanism, comparative transcriptome analysis of salinity tolerant and sensitive cultivars was carried out by Puranik *et al.* (2011) wherein a total of 159 differentially expressed transcripts were identified. However first systematic study to identify salt-responsive differentially expressed transcripts was carried out by Jayaraman *et al.* (2008) where 90 transcript derived fragments were identified using cDNA-AFLP technique in two foxtail millet cultivars, tolerant and sensitive for salinity tolerance. Earlier, Sreenivasulu *et al.* (2004) identified several differentially expressed transcripts in salt-treated tolerant and sensitive foxtail millet seedlings using a macroarray filter with 711 cDNA spots representing 620 unigenes of a barley EST collection.

Extensive genome-wide investigation and expression profiling studies of various TF families such as AP2/ERFs, NAC, C_2H_2 zinc finger and MYB (Lata *et al.*, 2014; Puranik *et al.*, 2013; Muthamilarasan *et al.*, 2014b; Muthamilarasan *et al.*, 2014c) and stress responsive genes such as WD40, DCL, AGO and RDR polymerases and ALDH (Mishra *et al.*, 2014; Yadav *et al.*, 2014c; Zhu *et al.*, 2014) have also been carried out in foxtail millet for analyzing gene families implicated in stress tolerance, identifying suitable candidate genes and delineating gene regulatory networks and molecular cross talks for abiotic stress responses.

Pearl Millet

Pearl millet is known for its drought and heat tolerance as it can withstand severe moisture and high temperature stress and can adjust a wide range of soil conditions. It is also being considered as a potential biofuel crop (http://ag.fvsu.edu/index.php/research/

bioenergy/). Yet until very recent times, pearl millet received comparatively little attention from the scientific community. Identification and characterization of stress responsive genes and their targets from pearl millet will not only help in understanding stress regulated pathways but will also help in designing strategies for improving stress tolerance/resistance of pearl millet as well as other related crop plants. There has been only one report on transcriptome analysis of pearl millet under abiotic stress conditions (Mishra *et al.*, 2007). A total of 2,494 differentially regulated transcripts in response to drought, salinity and cold stress were identified and the study indicated the existence of a complex gene regulatory network that differentially modulates gene expression under various stresses. Though its large genome size has been a hindrance for sequencing its genome, pearl millet comprehensive transcriptome has been developed by combining together the transcriptome data of three independent research works namely Rajaram *et al.* (2013), Zeng *et al.* (2011) and Yves *et al.*, (unpublished; http://www.ceg.icrisat.org/transcriptome.html). The transcriptome analysis of pearl millet under drought stress using next-generation sequencing (NGS) approach is also under progress and is thought to provide novel insights into gene regulatory networks operating in this naturally stress tolerant crop (Lata *et al.*, unpublished observations).

Finger Millet

Finger millet is also known for its superior tolerance against various abiotic stresses such as drought and salinity. Rahman *et al.* (2014) reported the identification of several salinity responsive genes and genotype specific responses from transcriptome analysis of contrasting finger millet genotypes differing for salinity tolerance through mapping and annotation of identified transcripts using rice gene models. In another important study, transcriptome wide identification and validation of calcium sensor gene family in the developing spikes of finger millet was carried out in order to understand their role in calcium accumulation (Singh *et al.*, 2014). Various transcriptomic studies carried out in these three millet crops till date is listed in Table 2.

Proteomics

The term 'proteome' refers to the entire set of proteins produced or modified by an organism, and 'proteomics' therefore refers to a large-scale analysis of proteins expressed at a given stage or condition in a cell, tissue or organism in order to understand the biology or underlying regulatory mechanism. The existent state of any organism is indicated by its proteome which is essentially a link between its transcriptome and metabolome. As mRNA levels may not be always correlated with protein accumulation and also that proteins tend to act directly on biochemical processes, proteomics approach becomes rather essential in evaluating plant stress responses (Gygi *et al.*, 1999). In recent years, a number of novel approaches have been developed for deeper proteome analyses from particular tissues or subcellular fractions which generally complement the well-established two dimensional electrophoresis (2-DE) technique (Barkla *et al.*, 2003). Post-translationally modified proteins are generally identified using mass spectrometry (MS)-based proteomic approaches (Barkla *et al.*, 2003). Despite the advancements in proteomics research, reports on proteomics application to crops are scanty as compared to other biological systems (Reddy *et al.*, 2012). Furthermore, as compared to transcriptome analysis, proteomic analysis in plants in response to various stresses is still very limited (Canovas *et al.*, 2004) with only one study reported in foxtail millet among all millets. Proteomic analysis of 7 day old salt-treated tolerant cv. 'Prasad' seedlings was carried out using two-dimensional electrophoresis which led to the identification of 29 differentially expressed proteins involved in signal transduction, photosynthesis, metabolism and stress response (Veeranagamallaiah *et al.*, 2008). However protein profiling along with SSR and RAPD-PCR of 52 finger millet genotypes has also been reported which was carried out in order to investigate the variation in protein content (Kumar *et al.*, 2012).

Metabolomics

Genomics, transcriptomics or proteomics have undoubtedly played important role in providing information regarding genotype and complex biological

Table 2: Details of transcriptomic studies carried out in foxtail millet, pearl millet and finger millet under different abiotic stresses

Crop	Strategy	Nature of study	Reference
Foxtail millet	Illumina HiSeq 2000 platform	Transcriptome analysis of 14-day old var. Yugu1 seedlings exposed to 20% Polyethylene glycol (PEG) 6000 stress at different time points Tissue: Shoot	Qi *et al.*, 2013
	Suppression subtractive hybridization (SSH) and dot blot array	Comparative transcriptome analysis of contrasting foxtail millet cultivars under short-term salinity stress (250 mM NaCl; 6 h) Tissue: Whole seedling	Puranik *et al.*, 2011
	SSH and dot blot array	Comparative transcriptome analysis of a tolerant foxtail millet cv. Prasad at early (0.5 h) and late (6 h) time points of 20% PEG-induced drought stress Tissue : Whole seedling	Lata *et al.*, 2010
	cDNA-AFLP	Comparative cDNA-AFLP analysis between contrasting foxtail millet cultivars differing for salinity tolerance Tissue: Leaves	Jayaraman *et al.*, 2008
	SSH and microarray	Comprehensive transcriptome analysis of a tolerant foxtail millet cv. Mar1 under drought stress (20% PEG) Tissue: Root and shoot	Zhang *et al.*, 2007
	cDNA macro array	A barley cDNA macroarray used for monitoring transcript abundance in contrasting foxtail millet seedlings exposed to long-term high salinity stress Tissue: Whole seedling	Sreenivasulu *et al.*, 2004
Pearl millet	Illumina MiSeq platform (2X300 bp chemistry)	Comparative trancriptome analysis of two contrasting pearl millet genotypes (parents of a mapping population) differing for terminal drought tolerance Tissue: Leaves	Lata *et al.* unpublished
	Subtracted cDNA library	Identification of differentially regulated transcripts from a drought tolerant parental line under salinity, drought and cold stresses Tissue: Leaves and roots	Mishra *et al.*, 2007
Finger millet	Illumina HiSeq 2000 platform	Transcriptome sequencing of two contrasting finger millet genotypes Tissue: Four stages of developing spikes	Kumar *et al.*, 2014
	Illumina HiSeq 2000 platform	Transcriptome sequencing of two finger millet genotypes differing in grain calcium content Tissue: Spikes	Singh *et al.*, 2014
	IonProton platform	Sequencing of salinity responsive leaf trancriptome of two contrasting finger millet genotypes Tissue: Leaves	Rahman *et al.*, 2014

processes but still are insufficient in discerning phenotype which is ultimately determined by a cell's metabolite. Therefore among all 'omics' sciences, 'metabolomics' is one of the newest. 'Metabolome' in particular refers to the complete set of low molecular weight compounds present in a sample which are either substrates or byproducts of enzymatic reactions and tend to have a direct effect on phenotype of a cell/tissue/organism. Metabolomics thus aims at determining a sample's metabolites profile at specific time or stage or environmental conditions and hence provides a straight functional statement of an organism's physiological condition. Further, transcriptome or proteome do not necessarily relate

to changes in the cell metabolome, thus emphasizing the relevance of metabolomics in the functional genomics era (Sumner *et al.*, 2003). Various conceptual approaches in metabolomics include target analysis, and metabolite profiling and fingerprinting that can be eventually used for several large-scale applications such as phenotyping transgenics, gene function determination, substantial equivalence testing, and monitoring stress responses. Metabolomics is thus considered to bridge the gap between genotype and phenotype. Therefore an integrated analysis of transcriptome, proteome, and metabolome is essential to have a complete understanding of both gene function and molecular events regulating complex biological processes. Most of the metabolomics work has been carried out in model plants and staple cereals with no report of metabolic profiling in any of the major millet crops. There is only one report of metabolic profiling in three varieties of proso millet using gas-chromatography-time-of-flight mass spectrometry (GC-TOFMS) to determine diversity among primary metabolites and phenolic acids (Kim *et al.*, 2013).

Phenomics

'Phenomics' is an equally important 'omics' technology critical for advanced genomics. The term phenomics refer to measurement of 'phenomes' or more precisely physical and biochemical traits of an organism as they tend to change under the influence of environmental stresses or genetic mutation. In order to harness the wealth of information generated by genomics platform has to be precisely linked to the phenotype. Infact unless phenotypic expression of plants displaying any level of genomic/trancriptomic/proteomic/or metabolic profile is not accurately characterized or understood, calling any of the above approaches successful will be wrong. A large gap exists between the knowledge of genotype and phenotype or in other words linkage between genotype and phenotype is rather illusive (Furbank and Tester, 2011). Indeed high throughput physiology and phenotyping has emerged as a new bottleneck in plant breeding and stress biology (Yang *et al.*, 2013). Therefore it is necessary to formulate certain basic principles for phenomics characterization for detailed analyses of any abiotic stress in crop plants. Rigorous phenotyping layout in the form of dry-down technique for progressive water stress has been developed in pearl millet in addition to WUE and drought resistance index (Vadez *et al.*, 2012). Other than these tillering, flowering time and rooting ability have also been the primary criteria for phenotyping in pearl millet (Vadez *et al.*, 2012). In foxtail millet, LP has been established as an important marker for oxidative stress (Lata *et al.*, 2011b). Though foxtail millet also needs development of efficient phenotyping methods both high throughput and manual for designing effective breeding strategies considering its importance as an excellent model system for studying biofuel crops.

Bioinformatics and Systems Biology Approaches for Omics Research

Understanding complex regulatory networks and the biological principles governing abiotic stress responses and tolerance in plants require sound knowledge of genome-scale responses at various developmental stages as well as against different environmental stimuli. Mostly expression datasets are available for model plants such as *Arabidopsis* and rice. Mining such datasets usually depends on various clustering algorithms that find groups or clusters of coexpressed genes that are hypothesized to be co-regulated under same internal or external stimuli by similar set of transcription factors and hence tend to form a transcriptional sub-network (Moreno-Risueno *et al.*, 2010). This principle can be used to infer regulatory hierarchies of gene expression across different species and genera. Further analysis of more complex and large-scale gene expression data require additional sophisticated approaches wherein bioinformatics tools play an important role. Using advanced bioinformatics tools and pipelines genome-scale data from various time-course experiments under diverse environmental conditions can easily be processed to identify and infer significant patterns coupled with specific *cis*-elements and regulatory such as TFs as well as other stress/stimuli-specific genes, and thus help in reconstructing regulatory and metabolic networks involving them and their regulatory interactions (Ma *et al.*, 2007; Moreno-Risueno *et al.*, 2010).

As far as proteomics and metabolomics are concerned, proteomics uses mass spectrometry (MS) based studies for protein profiling while metabolomics uses MS or nuclear magnetic resonance (NMR) based studies for metabolite profiling of particular cells, tissues, organelles/organs or whole plant. The data obtained from these two studies can be correlated with transcriptome profiles to decipher various signaling and metabolic networks for a particular internal or environmental cue. Metabolome studies in particular have the potential to elucidate biochemical networks, however integration of unknown metabolites into such networks is not possible by simply correlating analyses as in case of transcriptional or proteome networks. In such cases an integration of QTL analysis and qualitative metabolite profiling may be helpful in establishing *de novo* models on biochemical networks involving complex plant responses (Rowe *et al.*, 2008). A description of large-scale omics data integration for the reconstruction and analysis of biological networks and pathways is shown in Fig. 3. Until now not much work has been done using systems biology approaches for deciphering regulatory networks in millet crops, and therefore it is critical to make rigorous efforts in this direction by researchers worldwide to understand the biological networks operating in these otherwise nutritionally and economically important orphan crops.
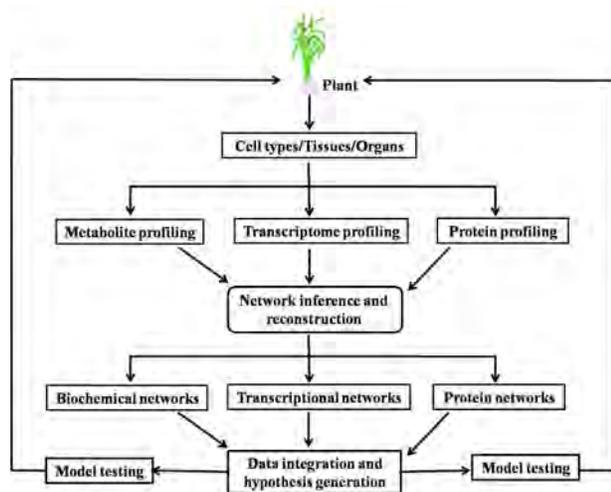

Fig. 3: A depiction of large-scale omics data integration for the reconstruction and analysis of biological networks and pathways

Conclusion and Future Perspectives

In recent years, molecular biology and biotechnology has appeared as a promising tool for overcoming stresses in plants, however, to date the progress has been limited in millet crops. Omics approaches have proved to be the most straightforward and potential biotechnological applications for improving abiotic stress tolerance in millets which lack proper resources for conventional plant breeding. Nevertheless successful application of omics for abiotic stress tolerance depends on the knowledge of stress response at molecular level. Therefore, with the availability of foxtail millet genome sequence, and ongoing genomics program in pearl millet and finger millet, application of omics for abiotic stress research in millets will be manifold. The current and up-coming high-throughput sequencing platforms are able to provide a wide variety of sequencing applications to researchers including identification of small RNAs, SNPs and molecular markers discovery. NGS approaches together with genome-wide expression profiling studies will be able to overcome the problems put forward by large genomes particularly those like millets. As compared to genomics and transcriptomics, proteomics and metabolomics studies still lag behind in millet crops. However with the advancements in high-throughput proteomics and metabolomics approaches such as flow injection – time-of-flight mass spectrometry etc., as well as an integration of various omics strategies will revolutionize the study of complex biological systems such as those of millets. It will not only help in large-scale identification of genes/proteins and metabolites involved in multiple molecular and signaling networks but also enhance our fundamental understandings on the molecular basis of abiotic stress tolerance in these crops. Hence, it is essential to extensively carry out these kinds of studies in millets for overcoming abiotic constraints. A concerted effort including all omics will be an important step towards deciphering abiotic stress tolerance mechanism in millets which can further be utilized for MAS or conventional breeding. Mapping of abiotic stress QTLs in millet crops is still in an early stage with no report on gene pyramiding till date. Availability of more number of ESTs and genome sequences will not only play important role in translating the knowledge in

other millet crops and grasses but also in QTL mapping and other breeding strategies. Genetic engineering of millet crops for improved abiotic stress tolerance should also be a focal point of research which will only be possible by identification of more number of potential candidate genes. Besides it is also important to have a careful and meaningful phenotyping for understanding the mechanisms of stress adaptation before identifying and tagging genes thought to be responsible for such mechanisms. Nevertheless current advances in omics technologies together with advances in transgenic technology and MAS will prove useful in improving the present scenario and it will indeed be possible to target all millet crops for genetic improvement.

Acknowledgements

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