



Recent advancements and future perspectives of foxtail millet genomics

Anurag Panchal¹ · Roshan Kumar Singh¹ · Manoj Prasad¹

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Abstract

One of the oldest domesticated crops, foxtail millet (*Setaria italica* (L.) P. Beauv.) is known for its enormous seed production and ability to grow in unfavourable agro-economic conditions. This understudied diploid panicoid crop plant which performs C₄ type of photosynthesis, contains approximately seven folds higher proteins with macro and micro-nutrients as compared to major cereals including rice and wheat. Encompassing a small genome size of ~515 Mb, short life cycle, and inbred properties make this orphan crop a potential model system to study abiotic stress tolerance mechanisms adopted by plants. Rich phenotypic variation and the largest available germplasm collection of cultivated and wild species exists for foxtail millet. Release of whole genome sequence in 2012, led to acceleration of functional genomic studies and molecular characterization of *Setaria* genes conferring stress tolerance. Recent advancements in integrative OMICS and NGS approaches are contributing in functional analysis, understanding complex gene regulatory networks and molecular mechanisms behind its growth and development. Abiotic stress tolerance traits of foxtail millet including drought, salinity, and its survival in nutrient-poor soil can be introduced in popular crop plants by genetic engineering techniques. Stable plant transformation system and precise gene editing in foxtail millet are under optimization which can revolutionize the theme of climate resilient future crops. Overall, foxtail millet has characteristics to serve as an excellent C₄ model plant to study evolution, stress physiology and biomass production for bioenergy crops to serve sustainable global food security in near future.

Keywords Abiotic stress · C₄ photosynthesis · CRISPR/Cas9 · Climate resilient crop · OMICS · WUE · NUE · Food security

Introduction

Agronomically, Poaceae is a predominant plant family of cereal crops, which have been used as a major staple food by the global population since ages. Out of this, Panicoideae is the second largest subfamily comprising over 3,500 species including maize, sugarcane, sorghum, and millets (Soreng et al. 2015). Millet is a universal terminology used to define cereal crops which produce small-sized but abundant seeds. Large proportion of millets with economic importance are

used for biofuel and bioenergy purposes. Foxtail millet (*S. italica*) is a grass which in recent years has emerged as a C₄ model crop plant for genetic studies (Singh et al. 2021). Plant is generally 120–200 cm in height, has a small genome size of about ~515 Mb, short life cycle (10–14 weeks), and produces a bountiful number of seeds as high as 13,000 per plant. Thin stem ends with a bristle-like panicle of about 5–30 cm with reddish or purplish color. This distinct appearance of the panicle looks like a fox's tail earning the popular name “foxtail” for most of the cultivated *Setaria* members. Foxtail millet performs NADP malic enzyme type C₄ photosynthesis. Including high photosynthetic efficiency, foxtail millet is also attributed with its higher WUE (Water Use Efficiency) and NUE (Nitrogen Use Efficiency) during unfavorable conditions leading to high yield and productivity even with low input resources (Brutnell et al. 2010). Not many detailed studies have been conducted on foxtail millet as compared to major cereal crop plants like rice, wheat,

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✉ Manoj Prasad
manoj_prasad@nipgr.ac.in

¹ National Institute of Plant Genome Research (NIPGR), Aruna Asaf Ali Marg, 110067 New Delhi, India

and maize. This was because of less functional genomic and genetic resources available to dissect the role of potential foxtail genes in imparting stress tolerance. This review focuses on origin, evolution, germplasm collection, genome sequencing, association studies, and recent advancements in functional genomics of foxtail millet with a glimpse of future goals to use foxtail millet in crop improvement for food security to serve the world with changing climate.

Origin, evolution, and genomic diversity of foxtail millet

Foxtail millet is the second highest cultivated millet after pearl millet across the globe. It belongs to the genus *Setaria* comprising approximately 125 species which are spread in mostly warm and temperate regions of the world. *S. italica* and *S. viridis* encompasses AA type of genome with $2n=2x=18$. Tetraploid weeds of this family, *S. faberii* and *S. verticillata* are comprised of AABB type of genome which are thought to be result of a cross between *S. viridis* and *S. adhaerans*, which is a diploid (Benabdelmouna et al. 2001). A CC type diploid species *S. grisebachii* was identified from Mexico by Wang et al. (2009). Another unique autotetraploid member of foxtail family is *S. queenslandica*, with AAAA genome. Modern day *Setaria* species including cultivated forms are thought to have originated from their common wild ancestor *S. viridis* which is also known as green foxtail millet. Almost more than 100 different *Setaria* species were domesticated for agriculture purposes. Nevertheless, only *S. italica* became a major staple cereal crop plant and became a part of ancient Chinese civilization. As per archaeological evidence, sites near the middle region of the Yellow river, Shanxi (Northern China) were the first places where the domestication was reported (Lu et al. 2009; Doust et al. 2009). Prime centres for evolution and subsequent diversification were East Asia, Japan and China, respectively. Another exploration hypothesizes the independent cultivation of *Setaria* species in arid and dry regions of Middle East Asia and Europe since 4000 BC. Studies using ribosomal DNA, phenotypic variation and isozymes proved that these landraces were more congenial with foxtail millet as compared to other regions of the world (Diao and Jia 2017). D'Ennequin et al. (2000) suggested a monophyletic origin of *S. italica* and *S. viridis* through AFLP loci data supported by the presence of distinct genotypes in China. However, Fukunaga et al. (2002) through RFLP analysis reported the nature of this evolution to be polyphyletic. At present, foxtail millet is scattered among US, Canada, some regions of India, China, Japan, Peninsula Korea, Australia, Indonesia, and a few northern parts of Africa (Doust et al. 2009; Li and Brutnell 2011). *Setaria* genus shares its

common ancestry with biofuel crop plants like pearl millet (8.3 MYA), proso millet, and switchgrass (13.1 MYA). Whole genome duplication event in the entire grass family is evident before separation of foxtail millet from sorghum and maize (27 MYA). Although, foxtail millet, yellow foxtail, and *S. macrostachya*, were domesticated for their usage as cereals, only foxtail millet became a global crop, and thus bestowed to the development of human civilization for more than 10,000 years in most regions of Eurasia.

Genetic relationship of foxtail millet with other graminaceous crops

Conserved collinearity studies, upon comparison of *Setaria* with genomes of grasses including sorghum and rice show extensive synteny (Zhang et al. 2012). Sorghum showed fewer chromosomal rearrangements when compared to rice. This study concludes the introduction of genetic variation between these close relatives due to chromosomal shuffling events and ultimately leading to speciation (Bennetzen et al. 2012; Zhang et al. 2012). Upon comparison of foxtail millet with sorghum, rice, Brachypodium, and maize, respectively, results indicated 71.8, 72.1, 61.5, and 86.7% similarity between the genomes (Muthamilarasan et al. 2017). Three rice chromosomes got independently fused to form three foxtail millet chromosomes. Out of those three, two chromosomes during evolution assimilated in sorghum, demarcating chromosome fusion events which had occurred before their divergence 27 MYA (Zhang et al. 2012). Presence of conserved blocks of collinearity, indicated rare presence of chromosomal fusions in graminaceous crops. In addition, comparative genomic studies using mapping approaches at gene family and marker hierarchy were also reported. First comparative mapping with functional markers was attempted by Pandey et al. (2013) of developing SSR markers which was followed by Kumari et al. (2013) using EST (Expressed Sequence Tags) derived SSRs and Muthamilarasan et al. (2014) for intron length polymorphic (ILP) markers developed to study synteny between foxtail and related crop plants. Similar studies confirmed higher synteny between foxtail millet and sorghum, succeeded by foxtail millet and maize through molecular marker studies. Further, comparative mapping of protein-coding genes involved in cellulose synthesis revealed maximum homology of foxtail millet with switchgrass genome. A brief study by Kong et al. (2019) reveals the phylogeny of sugar transporter genes in Gramineae (Poaceae) crops. This study concludes that during the course of evolution, sugar transporter protein family remains conserved and falls under high purifying selection pressure. Release of draft genome sequence, promoted in exploring and comparison of gene

families present in crops like Brachypodium, sorghum, rice, and maize with *Setaria* species.

Genetics and genomics resources of foxtail millet

Germplasm collection for foxtail millet

Primary and most vital step for understanding genetic diversity of a crop is its germplasm collection from diverse regions. The National Institute of Agrobiological Sciences, Tsukuba, Japan has around 1,300 accessions with entries from many other countries as well. These collections were classified by Dekaprelerich and Kasparian (1928) into two groups namely, Moharium and Maxima. Moharium had smaller panicles with numerous tillers/branches. Contrastingly, Maxima had larger panicles with only single/fewer tillers/branches. Later, Rao in 1987 added another group, Indica in between Moharium and Maxima in the classification. Inside this added group, 10 subraces namely *aristata*, *fusiformis* and *glabra* were placed in Moharia; *spongiosa*, *compacta*, and *assamense* in Maxima; *glabra*, *erecta*, *nana* and *profusa* positioned in Indica (Diao and Jia 2017). China has the biggest foxtail millet germplasm collection containing around ~28,000 accessions stored in Chinese Gene Banks. Representative core and mini-core collections of foxtail millet accessions have been developed by Krishnamurthy et al. (2014) and Upadhyaya et al. (2009, 2011). International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), India holds ~1,535 cultivated germplasm collections from around 26 different countries (Upadhyaya et al. 2009). This core collection includes potential resources for traits such as disease resistance, climate resilience and salinity stress tolerance (Krishnamurthy et al. 2014).

Genome sequencing and available genomics resources

Genome sequencing of foxtail millet was an independent effort of two globally known groups including Beijing Genome Initiative (BGI), China (Zhang et al. 2012) and the United States Department of Energy-Joint Genome Initiative (USDOE- JGI), USA (Bennetzen et al. 2012). Foxtail millet cultivar Zhang gu and A2 accession were used by BGI for Illumina second generation-based sequencing approach. The draft genome thus produced, predicted a genome size of ~485 Mb with approx. 6.6% gaps (~28 Mb) (Zhang et al. 2012; Lata and Prasad 2013). The JGI of USDOE used A10 accession which is *S. italica* inbred Yugu1 and *S. viridis* accession using ABI 3730xl capillary sequencer and were

Table 1 Tabular representation of major databases dedicated to foxtail millet genomics

Name of the database	Developed for	Reference	Link
SiGDB	Basic sequence search and alignment	(Duvick et al. 2007)	https://www.plant-gdb.org/SiGDB/
FmMDb	Molecular markers	(Bonthala et al. 2013)	http://www.nipgr.res.in/foxtail.html
FmMiR-NADb	Micro RNA	(Khan et al. 2014)	http://59.163.192.91/FmMiRNADb/
FmTFDb	Transcription Factor	(Bonthala et al. 2014)	http://59.163.192.91/FmTFDb/
FmTEMDB	Transposable Element based Markers	(Yadav et al. 2015)	http://59.163.192.91/FmTEMDB/
SiFGD	Integrative genomics and proteomics database	(You et al. 2015)	http://structuralbiology.cau.edu.cn/SIFGD/

able to assemble 4,003,684,795 bp data (Bennetzen et al. 2012). Upon resequencing of A10 nuclear DNA ~3,500 Mb data with 7X coverage was generated. A large chunk of data generated corresponded to transposable elements (TEs) where LTRs were the most abundant. Draft genome of Zhang gu had ~46% while Yugu 1 contained ~40% TEs. This characteristic is analogous to Poaceae members with small genomes like rice (46%) and sorghum (62%) (Zhang et al. 2012; Bennetzen et al. 2012) as compared to members like wheat and maize which have larger genome size with major chunk (upto 80%) as TEs (Bennetzen et al. 2012). Presence of huge amount of TE corresponds to their contribution in genome evolution, evolutionary relationship, regulation of epigenome, *cis*- regulation of gene expression or with some unknown and underexplored roles. Sequence variations in different *Setaria* genomes sequenced at that time, helped in determining different agronomic traits (Lata and Prasad 2013). Even before the release of the foxtail millet genome sequence, genomic resources for instance, molecular markers including SSRs (Gupta et al. 2012), intron length polymorphic (ILP) markers (Gupta et al. 2011) were being developed. Integrative marker databases of foxtail millet have also been developed which can help plant breeders to coordinate between molecular biology and plant breeding science of *Setaria* (Table 1). Sequencing data of foxtail millet genome is being applied in novel gene family identification, construction of high-density linkage maps and molecular markers to generate genetic resources for future research.

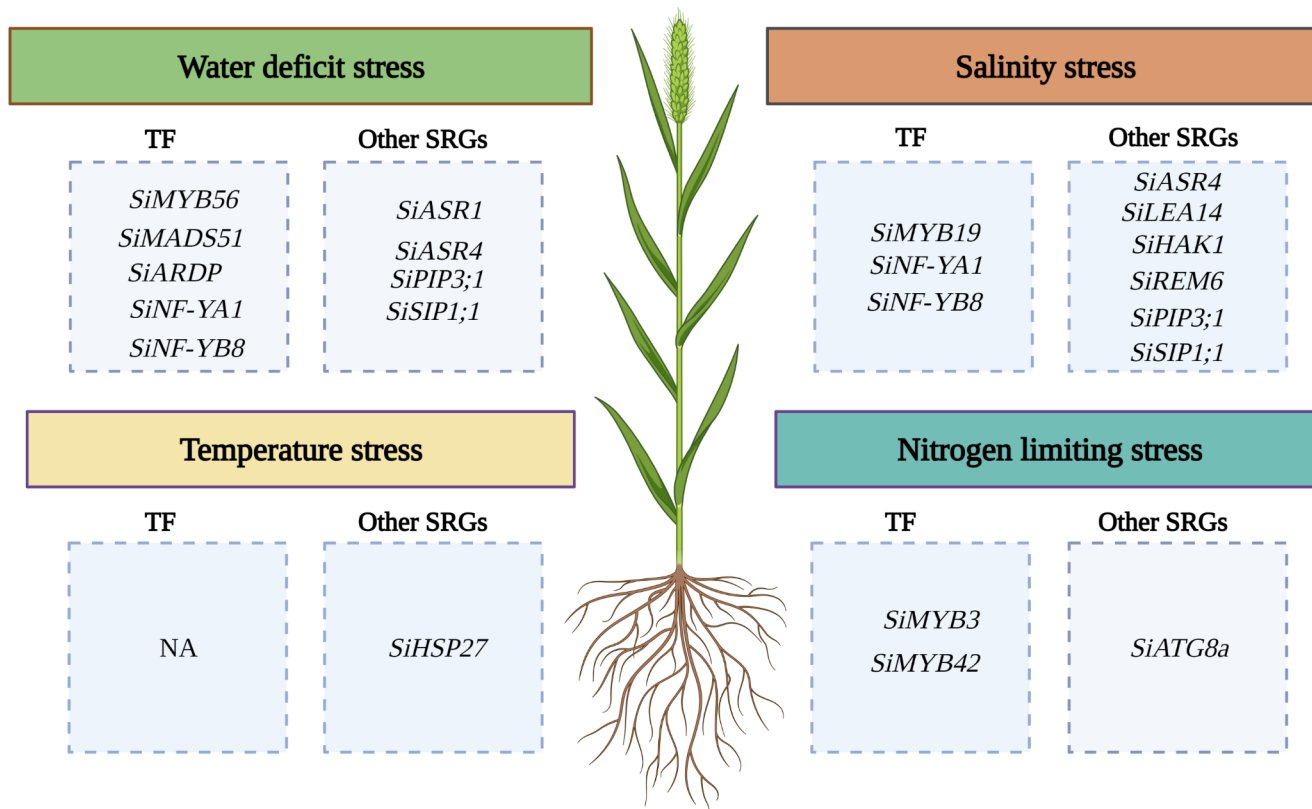


Fig. 1 Pictorial representation of functionally characterized genes in *S. italica* in the heterologous systems under water deficit stress, salinity stress, temperature stress, and nitrogen limiting stress. TF: Transcription Factors; SRGs: Stress Responsive Genes; NA: Not available. *SiMYB56* (Xu et al. 2020); *SiMADS51* (Zhao et al. 2021); *SiARDP* (Li et al. 2017); *SiNFYA-1* and *YB-8* (Feng et al. 2015); *SiASR1* and *SiASR4* (Feng et al. 2016); *SiPIP3;1* and *SiSIP1;1* (Singh et al. 2019); *SiMYB19* (Xu et al. 2022); *SiLEA14* (Wang et al. 2014); *SiHAK1* (Zhang et al. 2018); *SiREM6* (Yue et al. 2014); *SiHSP27* (Singh et al. 2016); *SiMYB3* (Ge et al. 2019); *SiMYB42* (Ding et al. 2018); *SiATG8a* (Li et al. 2016). (Image created using Biorender.com)

Functionally characterized abiotic stress-responsive genes and regulatory RNAs from foxtail millet

Rapidly changing environmental conditions greatly reduces the yield and productivity of crop plants. Plants being sessile organisms have to face a plethora of abiotic and biotic stresses during their life cycle. Hence, searching for new biotechnological or breeding approaches for crop improvement against stresses is a matter of concern globally. Although millets are well known for their inherent nature of being tolerant to various abiotic stresses, foxtail millet is well adapted to resisting drought, salinity, high temperature, and poor soil if compared to other cereal crop plants. The availability of genome sequence has boosted functional genomics studies in *S. italica*. Hence, discovering stress responsive genes from foxtail millet and their overexpression in major cereal crop plants seems a befitting strategy. Recent progress in the identification of such genes have been made to dig out their molecular function. A brief representation of

foxtail millet genes which have been functionally characterized so far using heterologous systems are shown in Fig. 1.

Drought stress tolerance

Foxtail millet has been reported to have higher WUE (Water Use Efficiency) than major crop plants like maize, sorghum, and wheat owing inherent tolerance to drought stress (Bandyopadhyay et al. 2017). However, severe sensitivity to drought stress can be seen at the inflorescence stage especially during the spikelet development stage (lasting about 35–50 days after sowing). Out of the total 1,517 millet specific gene families, foxtail millet specific 586 were envisioned to have roles in response to water and hence annotated as water responsible. A brief study by Wang et al. (2018) identified 149 foxtail millet Basic helix-loop-helix (*bHLH*) genes through genome-wide association study (GWAS) and confirmed induction of eight *bHLH* genes in response to drought stress. One out of these genes was found to be localized to a major quantitative trait locus (QTL) defining drought stress tolerance. This study hypothesizes

the role of these transcription factors in conferring drought stress tolerance to foxtail millet. Role of MADS-box TFs is well known for growth and developmental aspects of plants. For the first time, 72 different MADS-box genes have been identified in foxtail millet (Zhao et al. 2021). Quantitative RT-PCR (qRT-PCR) analysis of these TF genes showed significant induction of 10 *S. italica* MADS-box genes upon treatment with 15% polyethylene glycol 6000 (PEG 6000), 100 μ M abscisic acid (ABA), 100 mM NaCl, and 100 μ M gibberellic acid (GA) indicating the role of these genes in different environmental cues particularly water deficit stress. Upon overexpression in both rice and Arabidopsis *SiMADS51* showed reduced tolerance to drought stress followed by poor growth in drought stress conditions (Zhao et al. 2021). These experiments establish *SiMADS51* to be a potential negative regulator of drought stress. Recently, a drought induced R2R3-MYB transcription factor from foxtail millet, SiMYB56 has been identified. Overexpression of this TF gene in rice enhanced the drought stress tolerance levels by regulating lignin biosynthesis without growth penalties (Xu et al. 2020). Upon investigation, overexpression lines showed ABA accumulation in rice seeds. This overexpression of SiMYB56 in rice corresponded to upregulation of ABA synthesis and response related genes under drought stress. Similarly, many foxtail millet TFs and genes have been functionally characterized for their role in water deficit and drought stress. This includes ABA-responsive DRE-binding protein from *S. italica* (*SiARDP*) (Li et al. 2017), Nuclear Factors including NF-YA1 and NF-YB8 (Feng et al. 2015), Abscisic acid- stress- and ripening-induced genes *SiASR1* (Feng et al. 2016) and *SiASR4* (Li et al. 2017), plasma membrane intrinsic proteins *SiPIP3;1* and *SiSIP1;1* (Singh et al. 2019). Not just protein coding genes, microRNAs (miRNAs) have also been reported in plants to help withstand stressed conditions. Overexpression of *SimiRNA396* conferred drought resistant phenotype in transgenic Arabidopsis at seedling and mature plant stages, respectively (Geng et al. 2021). Transcription analysis of these Arabidopsis seedlings showed the effect of *SimiRNA396* on different stress responsive genes. These studies clearly suggest the prospective role of not just protein coding genes but miRNAs in positively regulating drought stress tolerance in plants.

Salt stress tolerance

Very recently, *SiMYB19*, a R2R3-MYB transcription factor from foxtail millet, have been reported to confer salt stress tolerance in transgenic rice (Xu et al. 2022). Overexpression of *SiMYB19* resulted in increased grain number, shoot height, and salt tolerance to transgenic rice in both field grown as well as salt pond grown conditions. Similarly, high

affinity K⁺ transporter of *S. italica* SiHAK1 and remorin protein gene *SiREM6* have been accredited with their critical roles in salt stress (Zhang et al. 2018; Yue et al. 2014). In another study, a family of RLKs (Receptor-Like Kinases) *PTII* (Pto-interacting 1) have been identified in foxtail millet (Huangfu et al. 2021). During this analysis, 12 *PTII* genes were identified. When given salt and oxidative stress, these genes were found to be differentially expressed upon their analysis through qRT-PCR. Out of these 12 genes, one gene *SiPTII-5* was overexpressed in heterologous hosts Yeast and *Escherichia coli*. Enhanced salt stress tolerance in the form of better growth was observed in both of the hosts *in vitro*. An atypical *Setaria* late embryogenesis abundant (SiLEA) protein upon overexpression exhibited improved tolerance to salinity stress in transgenic Arabidopsis and foxtail millet (Wang et al. 2014). Regulation of salt uptake in plants is facilitated through Calcineurin B-Like proteins (CBLs) having members like salt overly sensitive 3 (*SOS3*). Their role in imparting salt stress tolerance is being dissected out from plants like Arabidopsis, maize, rice, poplar, and sorghum. According to existing reports, CBLs and CIPK (CBL-Interacting Protein Kinase) may have a role in regulating stress tolerance in plants. Foxtail millet seedlings, when given different stresses including 250 mM NaCl, 20% PEG-6000, 10 μ M methyl viologen, 100 μ M ABA, and heat shock stresses showed induced expression of *SiCBL4* and *SiCIPK24* preferentially in young leaves (Zhang et al. 2017). *SiCBL4* was found to interact with *SiCIPK24* and recruit *SiCIPK24* to plasma membrane. Expression of *SiCIPK24* in transgenic Arabidopsis showed salt stress tolerant phenotype when compared to WT plants. Similar study with *SiCBL5*, showed enhanced salt stress tolerance in *SiCBL5* overexpressing *S. italica* plants. Overexpressing plants exhibited strong Na⁺ efflux and lower accumulation of Na⁺ ions in primary root cells as compared to RNAi lines with knocked *SiCBL5* expression (Yan et al. 2022). These studies suggest the possible role of salinity responsive genes of foxtail millet to be exploited in generating salt tolerant cultivars of major crop plants.

Nutrient starvation stress tolerance

Foxtail millet has an inherent capability to grow in nutrient poor soil. Normally plants are affected by low nitrogen and phosphorus in soil because of their high demand for proper growth and development. To cope up with low nitrates and inorganic phosphates in soil plants adopt changes in their root structure. One study by Nadeem et al. (2020) shows lower levels of Indole Acetic Acid (IAA) and Cytokinin (CK) accompanied by enhanced levels of GA as a response to low nitrogen stress in foxtail millet. To follow with, upregulation of nitrate transporters (NRTs) *SiNRT1.1*, *SiNRT2.1*,

and nitrate assimilation-related protein (NAR) *SiNAR2.1* expression was coupled with enhanced nitrate influx in root cells. This was shown to be favored by nitrogen uptake. In nitrogen deficient foxtail millet roots more soluble proteins were accumulated. This increased nitrogen uptake was shown as a primary adaptive strategy deployed by foxtail millet roots to combat low nitrogen stress. Autophagy is a well-known process in plants and animals for its role in intracellular degradation and recycling of amino acids for protection from senescence and nutrient starvation. Upon analysis, 37 such autophagy-associated genes were identified in *S. italica* (Li et al. 2016). qRT-PCR data of foxtail millet seedlings when given nitrogen starvation stress for 24 hours, showed very high expression levels of *SiATG8a*. Potential role of *SiATG8a* in nitrogen starvation stress was further validated in heterologous host i.e. rice plants with *SiATG8a* overexpressed, which exhibited better growth in nitrogen starvation conditions. Additionally, some transcription factors are well known to play important roles in adapting to environmental changes in the rhizosphere region. One such TF family is MYB which is involved in secondary metabolite production, signaling, and biotic stress resistance. In low nitrogen conditions expression of MYB TFs is high as compared to other TFs. In a study, *MYB3* expression significantly increased the low nitrogen stress tolerance in transgenic Arabidopsis and rice plants on media plate and field grown conditions respectively (Ge et al. 2019). Alongside nutrients, heavy metal stress is a result of anthropogenic activities which have long term detrimental effects on both plant and environment. H₂S which in recent years have emerged as a gasotransmitter have been discovered to enhance the Cr³⁺ stress tolerance in foxtail millet by its interaction with calcium signaling (Fang et al. 2014).

Proteomics and metabolomics studies in foxtail millet

With the advancements in NGS technology, multiple OMICS approaches are emerging as a triumphant routine to study plant biology. Functional genomics of a crop plant, analysis of genes responsible for growth and development, components of stress response network for increasing yield in various crops are some key roles being dissected out nowadays with the help of comprehensive proteome and metabolome studies. Differentially expressed genes don't always correspond to functional proteins highlighting the role of post transcriptional modifications. Stage specific and stress specific proteome data helps one to understand the inventory of proteins participating in regulatory networks. One such study shows the involvement of 2,474 DEPs (Differentially Expressed Proteins) by quantitative proteomic

analysis of seedlings subjected to drought stress (Pan et al. 2018). After setting the stringency threshold, 351 out of the total were found to be having variable expression in significant amounts. Dataset included stress and defense responsive genes like LEA and ROS scavenging enzymes which were found in increased abundance. Unlikely, proteins including some RL Protein kinases, pentatricopeptide repeat containing protein, and aquaporins were found to be decreased. The transcriptomic profile of the respective proteins clearly demarcated the role of post transcriptional regulation of gene expression modules. Post-translational protein modification is also an essential aspect to regulate cellular function. Proteins may undergo several reversible changes including phosphorylation, acetylation, ubiquitination, sumoylation, and methylation to induce or suppress their activity (Singh and Prasad 2022). Phosphoproteomic profiling of contrasting salt-responsive foxtail millet varieties, An04 and Yugu2 identified several differentially-regulated phosphoproteins (DRPPs) associated with regulation of gene expression, signal transduction, ion transport, and other metabolic processes (Pan et al. 2021). The study revealed that signal perception and transduction capabilities of Yugu2 to be more rapid as compared to An04 when exposed to salinity stress. Dynamics of DNA methylation have also been reported to regulate the gene expression during dehydration stress in foxtail millet (Pandey et al. 2017; Singh et al. 2016). However, further research needs to be carried out to pinpoint the role of post-translational protein modification during abiotic stress response in foxtail millet.

To follow with, metabolome is also a viaduct between the genome and resulting phenome. In a study by Wei et al. (2021), Liquid Chromatography-Tandem Mass Spectrometry (LC-TMS) method was used for association study of 150 foxtail millet germplasms followed by identification and quantification of 330 different metabolites. After creating a MS² spectral tag (MS² T) library, highly reproducible 1335 metabolite signals were obtained. Variation in the environment reflects in the growth patterns and metabolite profile of a particular plant species growing at different geographical regions. This hypothesis exactly follows the terms with the results obtained in this study indicating differences in PCA (Principal Component Analysis) and different metabolite accumulation in Indian and Chinese foxtail millet accessions, respectively. To dig out the genetic basis of these differences, foxtail millet accessions were sequenced and mapped to the reference genome of foxtail millet. As a result of mGWAS, 973 SNPs were found for 157 metabolites corresponding to 237 quantitative trait loci (Wei et al. 2021). Significant association per metabolite in different categories using statistical tools was found, which resulted in mapping to 47 mGWAS hotspots. An integrative study of transcriptome and metabolome of drought stress treated

foxtail millet seedlings reveal highly enriched amounts of genes involved in biosynthesis of phenylpropanoids, hormone signal transduction and phenylalanine metabolism (Yu et al. 2020). Under PEG-induced drought stress, high cinnamic acid accumulated in germinating seedlings, while other phenolic compounds like p-coumaric acid, ferulic acid, sinapic acid, and caffeic acid were found in decreased amounts. Some phenolics establish the influence of these aromatic biomolecules on roots and shoot growth patterns. These kinds of studies help in understanding the role of metabolites in abiotic stresses like drought and patterns of plants to defend themselves by continuing their basic physiology and growth even in unfavourable conditions.

Database resources for foxtail millet

Genome sequencing of foxtail millet brought this crop plant back to limelight in 2012, to the plant science community for functional genomic studies. Multiple databases were developed in order to provide the sequence information of individual genes. Few important databases which can be used to retrieve the sequence information of foxtail millet are as follows:

Gramene: Curated, open source and an exclusive data repository for plants which are attributed as “cereal crops”. This database can be used to search foxtail millet genes for their sequence and structure, expression and regulation, transcript and proteins (<http://www.gramene.org/>).

Phytozome: A comparative genomics resource developed by JGI, US (Joint Genome Institute, US) known for its high quality assembled and annotated plant genomes (Goodstein et al. 2012) (<https://phytozome-next.jgi.doe.gov/>). Database has features to browse the genome, align sequences using BLAST and study transposable elements.

SiFGD: *S. italica* Functional Genomics Database, China, a platform to retrieve genome sequence, expression patterns, non-coding regulatory RNAs, proteome, and expressed sequence tags (EST) of foxtail millet (<http://structuralbiology.cau.edu.cn/SIFGD/>) (You et al. 2015).

Ensembl Plants: Integrative database for plants which helps in genome browsing, aligning sequences, exon intron clipping of foxtail millet genes (<https://plants.ensembl.org/index.html>).

A detailed list of databases developed for foxtail millet genomics are tabulated below in Table 1.

Genetic engineering and genome editing for functional validation of identified genes

Plant genetic alteration remains the master technique to decipher the role of genes responsible for traits of interest. Engineering a crop plant with the vector construct having our gene of interest has been tried in most of the agronomically important plants. Unlike major cereals, foxtail millet is considered recalcitrant for transformation and regeneration *in vitro* for tissue culture. Till a few years back, only immature inflorescence was being used as an explant which resulted in ~5% transformation efficiency (Wang et al. 2011; Liu et al. 2005). Some recent studies show the method and use of foxtail millet genetic transformation being used for functional analysis of genes. Characterization of foxtail millet PHT1 family phosphate transporters included *Agrobacterium*-mediated transformation (Ceasar et al. 2017). Shoot apices as explants were used to knockdown the expression of three PHT1 transporter family members. As a result, significant reduction in inorganic phosphate was observed in shoots and root tissues. Enhanced number in lateral roots and root hair along with complementation studies establishes the role of phosphate transporters in foxtail millet for proper uptake and distribution of phosphorus in cells. In another method, *Agrobacterium tumefaciens*-mediated genetic transformation of foxtail millet using seed as an explant for callusing shows ~27% transformation efficiency (Sood et al. 2020). This evident protocol utilizes secondary embryogenic calli which shows high competence for *Agrobacterium* cells over primary calli. One latest study on foxtail millet specific C-terminally encoded peptides (SiCEPs) using *Agrobacterium rhizogenes* claims 76% transformation efficiency to make transgenic hairy roots (Zhang et al. 2021). Method was used to overexpress *SiCEP3* in foxtail millet, as an observation transgenic root showed higher ABA accumulation than empty vector lines. To functionally characterize a gene, a mutagenized population serves as a new germplasm resource. Such mutant population are well known in Arabidopsis and rice. Recently, EMS induced mutant population of *S. italica* have been reported which have also resulted in identification of carotenoid biosynthesis gene *SiWPI* (Sun et al. 2019). Moreover, large build-up and relatively long generation time, are the key aspects which make functional studies on foxtail millet as C_4 model crop strenuous. A breakthrough finding by Yang et al. (2020), discovered a *S. italica* mutant called ‘*Xiaomi*’ that can complete 5–6 generations in an year (65 days) with ~29 cm height. This Arabidopsis-like generation cycle phenotype was a result of a point mutation in one *PHYC* gene of Jingu21 cultivar of foxtail millet which provides it an early heading phenotype. *Xiaomi* can also be used as a strikingly efficient transformation system with ~23% transformation efficiency. Overall,

this finding establishes *Xiaomi* as a potential C₄ model crop to study genes for specific traits and stress biology studies including NUE with multiple generations covered round the year.

With the advancements in genetic modification techniques, targeted genome editing is also budding as a revolutionary biotechnological tool for crop improvement. Being the most effective one, CRISPR/Cas9 technology includes a single gRNA (guide RNA) and a Cas9 endonuclease. Cas9 cuts after recognising PAM (Protospacer-Adjacent Motif) sequence resulting in double stranded breaks which induces homology directed repair pathway to get the damaged DNA bases repaired. This precise base editing was used by Liang et al. (2022) to generate single and multi-gene knockouts to produce herbicide tolerant mutant foxtail millet plants. Another base editing by CRISPR strategy in foxtail millet was exploited to generate double haploids for breeding to screen recombinant haploid plants in a fixed background. Haploid embryo induction through seeds was done to simply mutate *SiMTL* gene through CRISPR/Cas9 base editing (Cheng et al., 2021). Two guide RNAs were used to mutate two exons of *SiMTL* gene followed by *Agrobacterium*-mediated transformation of Ci846 cultivar of foxtail millet. These doubled haploid lines can be used for their high induction rate (HIR) by foxtail millet breeders for crop improvement by gene stacking approaches. Overall, genetic transformation protocols already exists but they need advancement to increase efficiency and stability of transformation. If achieved, functional genomics of foxtail millet would trigger understanding the roles of several trait specific genes responsible for understanding foxtail millet biology.

Understudied areas in foxtail millet genomics as compared to major cereals

Genome sequencing of this orphan crop was done a decade back but still very few genes have been explored. With advancements in genome wide association studies, transformation techniques, annotation of genes, and identification of new gene family foxtail millet genomic research is rapidly growing. Attempts have been made in various major crop plants to dissect the hormonal pathways which get modulated when plants encounter abiotic stresses. Dissecting these mechanisms in foxtail millet can lead to a better understanding of the roles of stress responsive genes. Additionally reproductive biology aspects including seed size, seed architecture, and panicle architecture which provide the agronomic traits are still understudied in *S. italica*. Majority of studies done so far on foxtail millet revolves around finding new gene families and gene expression profiles. Very

little attention has been given to molecular interaction patterns and gene regulatory networks responsible for foxtail millet to withstand harsh environmental conditions of heat, drought, salinity, and poor soil quality. Repetitive sequences as well as transposable elements in genomes contribute to sequence variation and gene structures. Major study which can be done is integrating the PAN-genome of *Setaria* with all genes present in this genus to analyse the entire genomic diversity of foxtail millet seems a promising approach.

On one hand, where functional genomics and abiotic stress biology of crop plants like rice and maize are blooming, these studies in foxtail millet are still in their preparatory phase due to lack of genetic modification techniques. A brief timeline chart in Fig. 2 shows the major landmark discoveries done so far in the field of foxtail millet genomics. Genome editing techniques like CRISPR/Cas9 in millet are still being optimized by various groups across the world which will boost up the forward genetics to understand the genes contributing to target traits. Integrative studies of metabolome, transcriptome, IONome including high throughput techniques like single cell RNA-seq will only be effective after genome annotation and data curation of foxtail millet. These advanced methods will pave the foxtail millet genomics to better understand its physiology in abiotic stress conditions.

Applications of foxtail millet genetics and genomics resource to design climate-smart crops

Extreme weather events predominantly including drought, heat, salinity, and flooding are making the rapidly increasing world population vulnerable to food security and nutrition. Climate change reflects in plant metabolism, physiology, carbon sequestration, and soil quality. Much of the efforts are being made to enhance the yield and productivity of crop plants, especially to the ones with low input but good harvest. Focus should be on making climate-resilient crop plants which have the property to adapt to the stressful environments by impeding resilience to soil/plant interface (Dhankher and Foyer 2018). Drought tolerance ability of foxtail millet is a synchronous result of high WUE, epidermal cell arrangement, and thick cell wall. Cultivation of crop plants in semi dry, dry or marginal lands can be initiated with introduction of overexpressing drought tolerance responsible genes from foxtail millet. To achieve this, classical breeding approach and genetic modification using biotechnology remains the major two methods. To save time and intense labour in breeding programmes, genetic engineering is thought to be a smart approach. One key property that foxtail millet offers is its ability to grow in low nitrogen

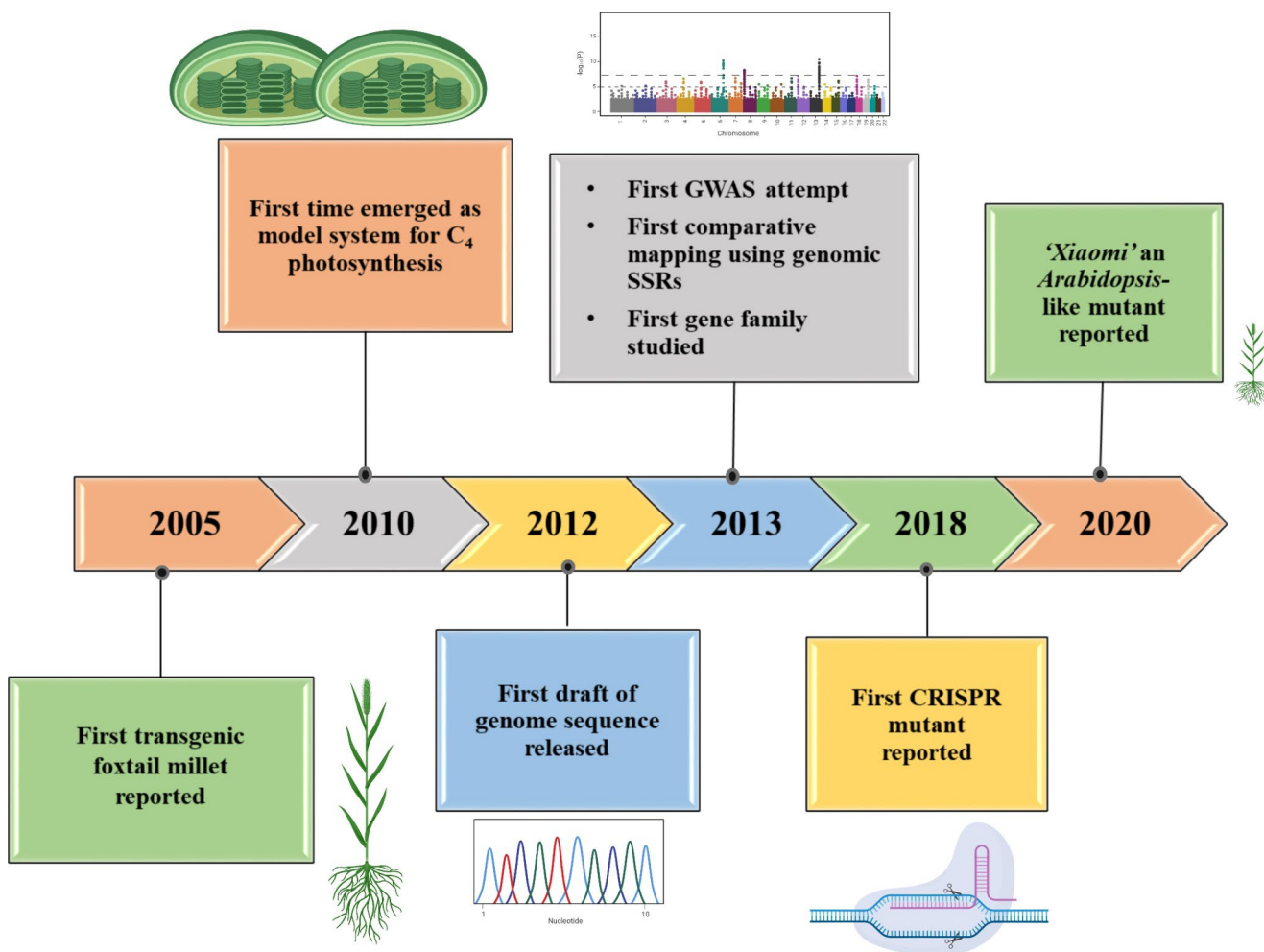


Fig. 2 Timeline chart showing landmark studies in foxtail millet genomics. (Image created using Biorender.com)

containing soils. Implicating lesser need of nitrate fertilizers for its growth in fields having high NUE (Bandyopadhyay et al. 2017; Bandyopadhyay et al. 2022). Furthermore, foxtail millet has a strong root system responsible for its survival in drought and low nutrient conditions. Crop plants can be engineered to introduce these properties provided by respective genes to make them more tolerant to changing climate conditions (see Fig. 3).

Yield of a crop plant is a result of its high photosynthetic efficiency. C_4 plants have been accredited to have better WUE and good harvest without much resources. Thus, engineering crop plants with C_3 traits to have C_4 anatomy would be a path-breaking strategy. One potent approach which have traditionally been used is forced hybridization of phylogenetically distant C_3 and C_4 plants (Simpson et al. 2022) to incorporate desired traits. This includes somatic and sexual hybridization techniques but these plants were either producing sterile flowers or the resultant hybrids were unstable after repeated rounds of cell divisions during embryogenesis. Attempts have also been made to

genetically transform C_3 plant to C_4 but no fruitful success was achieved. This has happened due to lack of ground level understanding of genes responsible for spatial separation of photosynthesis and kranz anatomy. However, a concerted effort by the plant science community can do the wonder of introducing C_4 rice and wheat, which will be a sustainable resource for food security.

Conclusion

Foxtail millet is an ancient, second largest cultivated crop with inherent tolerance to low fertility soil. It is a fantastic model system to study C_4 genetics, mechanisms to combat abiotic stresses like drought, salinity due to its high WUE and NUE. Huge germplasm collection of foxtail millet present across the globe is evident in representing the variety and large gene pool which can be used to establish their PAN-genome. Currently the large-scale data is being analysed using conventional statistical methods which have low

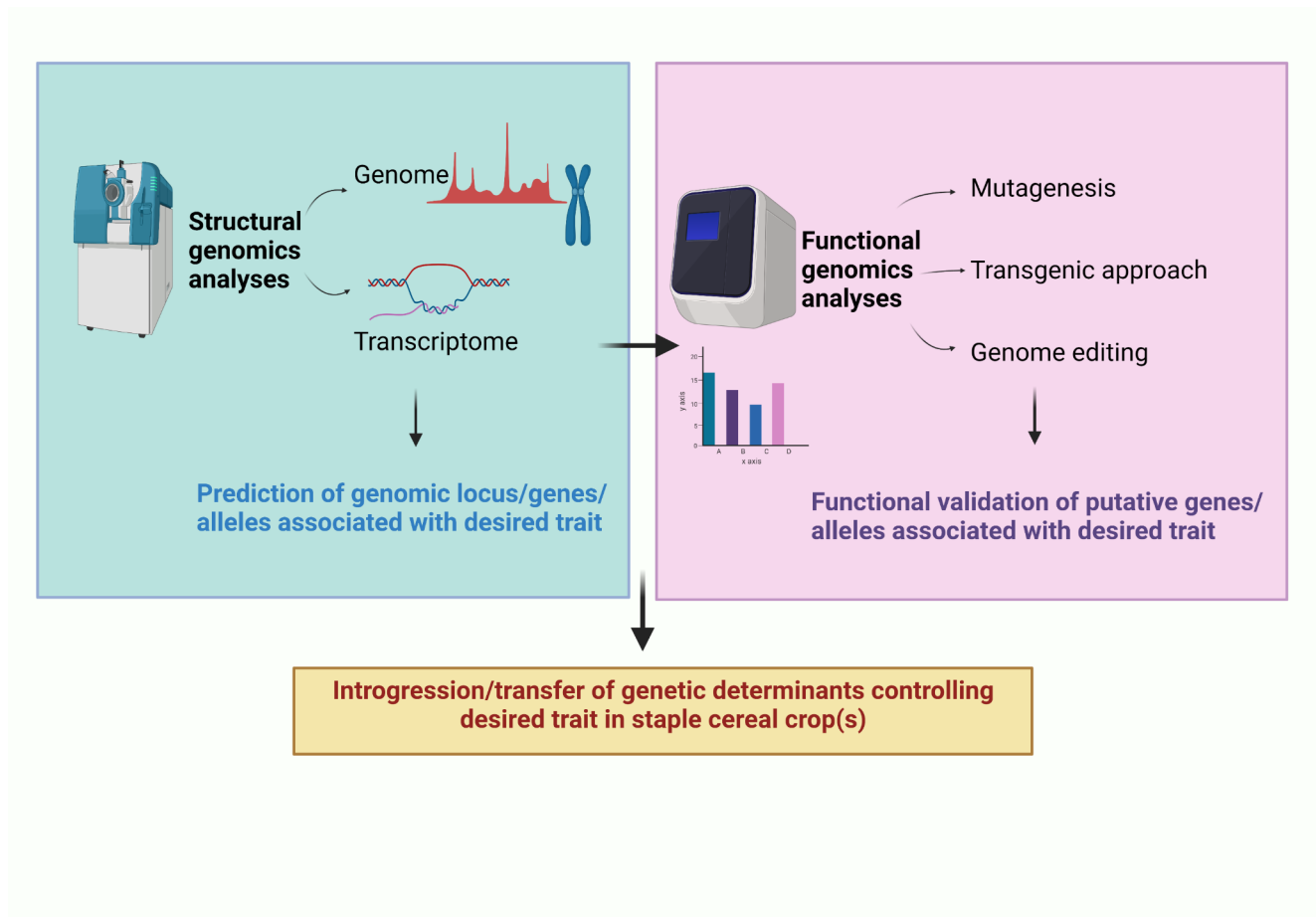


Fig. 3 Illustration of a brief pipeline for introgression/transfer of desired trait from foxtail millet in staple cereal crop plants. Structural genomics analyses help in prediction of genetic determinants for trait of interest. To follow with, functional genomics analyses help in understanding the regulation of genes responsible for traits which can be altered through mutagenesis, transgenic approach and genome editing, respectively. (Image created using Biorender.com)

efficacy. The adoption of machine learning would provide more precise analysis of phenotyping as well as genotypic characteristics in varied environmental conditions. Since the availability of genome sequence, functional genomic studies were accelerated resulting in characterization of various abiotic stress related genes participating in stress biology and biofuel traits. Foxtail millet, which once was a neglected and understudied crop is now considered as an excellent source of germplasm with several novel stress-responsive alleles for crop improvement. Recently, metabolome and proteome studies have depicted differentially expressed proteins and metabolites and their accumulation after an encounter with environmental stress. An integrated approach where the large-scale data from all the omics could be analysed together to dissect the master regulator of stress responses needs to be developed. Genetic dissection of C_4 physiology will help in designing C_4 crop plants which will pave the way for food security. Advancements in NGS technologies have opened the doors for allele mining,

SNP discovery in foxtail millet genotypes for breeding programmes. Advanced transformation and genome editing techniques will help in overexpression of stress tolerance genes and understanding development, physiology and stress tolerance biology in foxtail millet and cereal crops. Introduction of these genes/traits in major crop plants like rice and wheat to make climate resilient crops is the absolute necessity for future food security in global climate change.

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Authors' contributions AP, RKS and MP conceptualized and designed the outline of the manuscript. AP wrote the first draft of the manuscript. AP and RKS prepared the figures. RKS and MP revised the manuscript. All authors read and approved the final manuscript.

Declarations

Conflict of interest statement No conflict of interest declared.

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