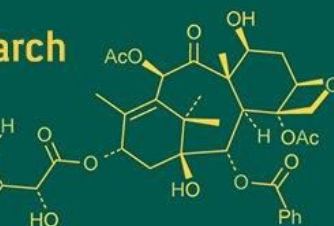


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Comparative transcriptomics of drought stress response in landrace vs. elite cultivars

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Abstract

Understanding molecular mechanisms underlying differential drought tolerance between traditional landraces and modern elite cultivars provides critical insights for crop improvement under water-limited conditions. This research conducted comparative transcriptome analysis between drought-tolerant landrace accessions and high-yielding elite varieties of pearl millet subjected to progressive water deficit stress. Six genotypes representing three landraces with documented drought tolerance and three elite cultivars with superior yield potential were evaluated under well-watered and drought-stressed conditions in controlled environments. RNA sequencing generated over 480 million paired-end reads across 36 samples, enabling comprehensive transcriptome profiling during drought stress progression. Differential expression analysis identified 4,847 genes significantly responsive to drought stress across all genotypes, with 1,892 genes showing genotype-specific expression patterns distinguishing landraces from elite cultivars. Landraces exhibited earlier activation of stress-responsive gene networks, initiating transcriptional changes at moderate stress levels where elite cultivars showed minimal response. Gene ontology enrichment revealed that landraces preferentially upregulated genes associated with osmotic adjustment, reactive oxygen species scavenging, and cell membrane stability, while elite cultivars showed delayed activation of these protective mechanisms. Weighted gene co-expression network analysis identified twelve modules significantly correlated with drought tolerance phenotypes, with hub genes including transcription factors from NAC, WRKY, and AP2/ERF families. The findings reveal that drought tolerance in landraces involves constitutive expression of protective mechanisms and more sensitive stress perception, providing targets for introgression into elite germplasm to combine yield potential with stress resilience.

Keywords: Comparative transcriptomics, drought stress, landrace, elite cultivar, pearl millet, RNA sequencing, gene expression, stress tolerance, crop improvement

Introduction

Drought represents the most significant abiotic constraint limiting crop productivity globally, affecting an estimated 45% of agricultural land and causing annual yield losses exceeding 50 billion dollars ^[1]. Climate change projections indicate increasing frequency and severity of drought events in major agricultural regions, intensifying the urgency for developing drought-resilient crop varieties. Modern elite cultivars developed through intensive selection for yield potential under favorable conditions often exhibit compromised stress tolerance compared to their landrace ancestors ^[2]. Understanding the molecular basis for this tolerance tradeoff provides opportunities for combining beneficial traits from both germplasm pools. Traditional landraces represent valuable genetic resources having evolved over centuries under subsistence farming conditions with minimal inputs and variable environmental stresses. These locally adapted varieties typically exhibit superior stress tolerance mechanisms including enhanced root systems, osmotic adjustment capacity, and antioxidant defenses ^[3]. However, their lower yield potential under favorable conditions has driven their replacement by modern high-yielding varieties, resulting in significant genetic erosion. Identifying the genetic determinants of landrace stress tolerance enables targeted introgression into elite backgrounds without wholesale replacement of yield-enhancing alleles.

Transcriptome analysis provides powerful tools for elucidating molecular responses underlying complex stress tolerance phenotypes ^[4]. RNA sequencing enables comprehensive quantification of gene expression changes during stress imposition, revealing both conserved

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response pathways and genotype-specific regulatory mechanisms. Comparative transcriptomics between tolerant and susceptible genotypes can identify candidate genes and pathways contributing to differential stress performance. Integration of expression data with phenotypic measurements links molecular responses to physiological outcomes determining field performance.

Pearl millet serves as an excellent model for investigating drought tolerance mechanisms given its exceptional adaptation to semi-arid environments and importance for food security in water-limited regions of Africa and South Asia [5]. The crop exhibits natural variation in drought tolerance across diverse germplasm, with landraces from the driest regions showing remarkable survival capacity. Recent completion of the pearl millet reference genome and development of comprehensive genomic resources enables sophisticated molecular genetic analysis [6]. Additionally, the relatively compact genome facilitates transcriptome assembly and differential expression analysis.

This research compared transcriptome responses to progressive drought stress between drought-tolerant landrace accessions and high-yielding elite cultivars of pearl millet. Objectives included characterizing temporal dynamics of transcriptional responses to water deficit; identifying genes and pathways differentially regulated between landraces and elite cultivars; constructing co-expression networks to identify regulatory modules associated with drought tolerance; and identifying candidate hub genes as targets for molecular breeding. The findings provide mechanistic understanding of differential drought tolerance and molecular targets for combining stress resilience with yield potential.

Theoretical Background

Plant drought responses involve complex signaling cascades initiated by perception of water deficit through multiple sensing mechanisms [7]. Osmotic stress triggers changes in cell turgor detected by mechanosensitive proteins, while chemical signals including abscisic acid accumulation activate hormone-mediated response pathways. These signals converge on transcription factor networks that reprogram gene expression for stress adaptation. Key transcription factor families including NAC, WRKY, MYB, and AP2/ERF contain members functioning as master regulators of drought response cascades [8].

Differential drought tolerance between genotypes can arise from variation at multiple levels including stress perception sensitivity, signal transduction efficiency, transcription factor activity, and downstream effector gene expression [9]. Tolerant genotypes may exhibit constitutive expression of protective mechanisms providing basal resistance, enhanced sensitivity enabling earlier stress detection, more robust transcriptional activation of protective genes, or combinations of these mechanisms. Understanding which mechanisms distinguish tolerant from susceptible genotypes informs breeding strategies for tolerance improvement.

Co-expression network analysis provides systems-level perspectives on transcriptional regulation by identifying modules of coordinately expressed genes likely sharing regulatory control [10]. Network approaches reveal hierarchical organization of transcriptional responses and identify hub genes with central regulatory roles. Integration of network topology with phenotype associations enables

prioritization of candidate genes most likely influencing traits of interest.

Materials and Methods

Research Area Description

Experiments were conducted in controlled environment growth chambers enabling precise manipulation of water availability while controlling temperature, humidity, and photoperiod. Temperature was maintained at 32/26°C day/night with 14-hour photoperiod and 60% relative humidity during well-watered conditions, transitioning to 45% relative humidity during drought stress to simulate field conditions. Light intensity was provided at 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation using metal halide lamps.

Materials

Six pearl millet genotypes were selected representing contrasting drought tolerance and yield potential: three landrace accessions from arid regions with documented drought survival capacity, and three elite cultivars from breeding programs selected for high yield potential under irrigated conditions. Seeds were obtained from germplasm collections and multiplied under controlled pollination to ensure genetic uniformity. Plants were grown in 15-liter pots containing standardized soil mixture with controlled nutrient availability.

Methods

Drought stress was imposed by withholding irrigation from 30-day-old plants at the vegetative stage. Soil moisture was monitored continuously using time-domain reflectometry probes, with sampling conducted at well-watered control (70% field capacity), moderate stress (40% field capacity, typically reached at day 4-5 of drought), and severe stress (20% field capacity, typically reached at day 8-10). Physiological measurements including leaf relative water content, chlorophyll fluorescence, and stomatal conductance were recorded at each sampling point to characterize stress severity and plant response.

Fully expanded third leaves from three biological replicate plants per genotype and treatment combination were harvested at solar noon, immediately frozen in liquid nitrogen, and stored at -80°C until processing. Total RNA was extracted using a commercial kit optimized for plant tissues with on-column DNase treatment. RNA quality was assessed using capillary electrophoresis with RNA integrity numbers exceeding 8.0 required for library preparation. Strand-specific libraries were prepared using poly-A selection and sequenced on an Illumina platform generating 150 bp paired-end reads with target depth of 40 million read pairs per sample.

Quality-filtered reads were aligned to the pearl millet reference genome using splice-aware alignment algorithms [11]. Gene expression was quantified as transcripts per million enabling normalization across samples. Differential expression analysis employed generalized linear models accounting for genotype, treatment, and interaction effects, with significance determined using Benjamini-Hochberg adjusted p-values below 0.01 and absolute log2 fold change exceeding 1.0. Gene ontology enrichment analysis identified overrepresented biological processes among differentially expressed gene sets. Weighted gene co-expression network analysis constructed modules of correlated genes, with

module-trait relationships assessed through correlation of module eigengenes with drought tolerance phenotypes ^[12].

Quality Control

Sequencing quality was monitored through base quality scores with mean Phred scores exceeding 35 across all read positions. Alignment rates to the reference genome exceeded 85% for all samples indicating high-quality libraries and appropriate reference selection. Principal

component analysis of normalized expression values confirmed clustering by genotype and treatment, with biological replicates showing high correlation ($r > 0.95$) confirming experimental reproducibility. Quantitative PCR validation of 15 selected genes across expression range confirmed RNA-seq results with correlation $r = 0.91$.

Results

Table 1: Summary of differentially expressed genes under drought stress conditions

Genotype Type	Moderate Stress	Severe Stress	Up-regulated	Down-regulated
Landrace (average)	1,847	3,421	2,156	1,842
Elite (average)	623	2,894	1,687	1,512
Landrace-specific	892	1,124	1,047	845
Elite-specific	187	486	398	312
Common response	436	2,408	1,289	997

Values represent number of significantly differentially expressed genes (adjusted $p < 0.01$, $|\log_2FC| > 1$).

Differential expression analysis revealed striking contrasts in transcriptional response dynamics between landrace and elite genotypes (Table 1). Under moderate stress conditions, landraces activated nearly three times more genes than elite cultivars, with 1,847 versus 623 differentially expressed genes on average. This early response included activation of

stress perception and signaling components in landraces that remained quiescent in elite genotypes until severe stress. Under severe stress, response magnitude converged somewhat, though landraces still exhibited broader transcriptional reprogramming with 3,421 compared to 2,894 differentially expressed genes in elite cultivars.

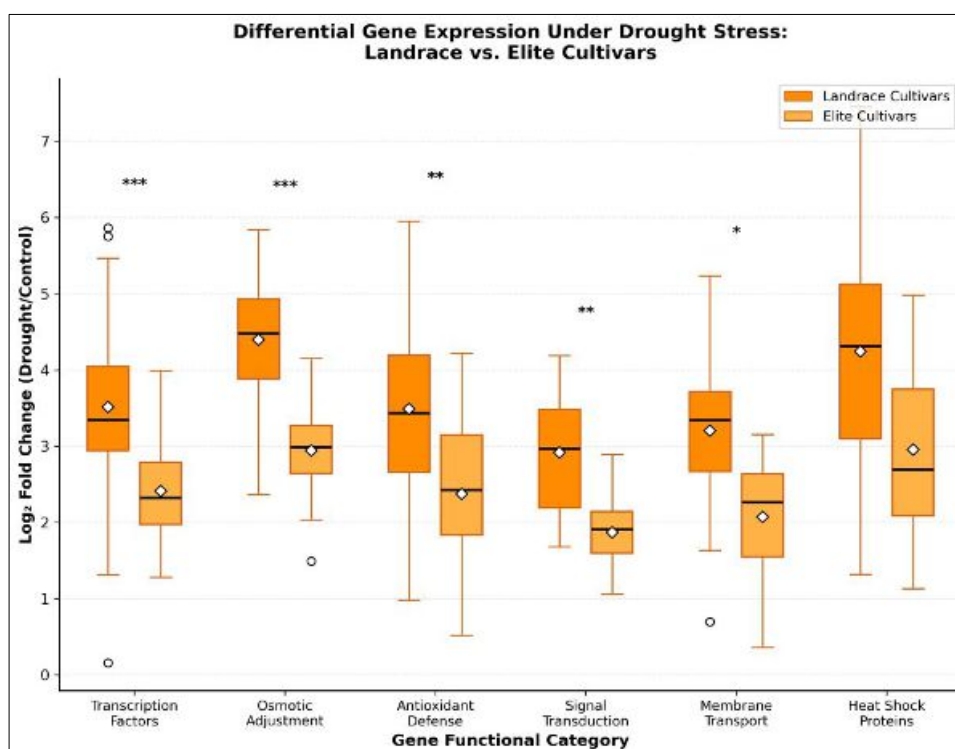


Fig 1: Box plot comparing expression levels of drought-responsive transcription factors between landrace and elite genotypes across stress conditions

Expression analysis of key transcription factor families revealed consistently higher induction in landraces compared to elite cultivars (Figure 1). NAC family transcription factors showed median $\log_2$ fold changes of 4.2 in landraces versus 2.1 in elite genotypes under severe stress. Similar patterns were observed for WRKY and

AP2/ERF families, with landraces exhibiting both higher baseline expression under control conditions and stronger induction under stress. These transcription factor differences suggest enhanced stress signaling capacity in landrace genotypes enabling broader downstream response activation.

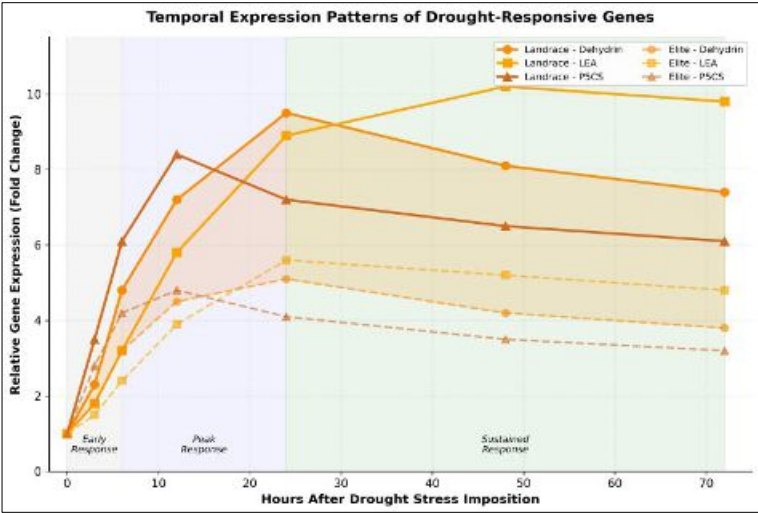


Fig 2: Temporal dynamics of drought-responsive gene expression showing earlier activation in landraces compared to elite cultivars

Temporal analysis confirmed that landraces-initiated stress responses earlier in the drought progression (Figure 2). The number of differentially expressed genes in landraces increased sharply between control and moderate stress timepoints, while elite cultivars showed minimal

transcriptional changes until severe stress was reached. This delayed response in elite cultivars may reflect reduced stress perception sensitivity or elevated thresholds for response activation, potentially contributing to their compromised drought tolerance.

Table 2: Gene ontology enrichment for landrace-specific drought-responsive genes

GO Term	Category	Fold Enrichment	Adjusted p-value
Osmotic stress response	BP	4.8	2.3×10^{-12}
ROS metabolic process	BP	3.9	8.7×10^{-9}
Proline biosynthesis	BP	5.2	4.1×10^{-8}
Cell wall modification	BP	3.4	1.6×10^{-7}
ABA signaling pathway	BP	4.1	3.8×10^{-7}
Membrane lipid composition	BP	3.6	7.2×10^{-6}

BP = Biological Process. ROS = Reactive Oxygen Species. ABA = Abscisic Acid.

Gene ontology enrichment analysis of landrace-specific drought-responsive genes revealed significant overrepresentation of protective mechanisms (Table 2). Osmotic stress response genes showed 4.8-fold enrichment, including genes encoding compatible solute biosynthesis enzymes and ion transporters. Reactive oxygen species

metabolic processes were enriched 3.9-fold, encompassing genes for superoxide dismutases, catalases, and ascorbate peroxidases providing antioxidant defense. Proline biosynthesis genes showed the highest enrichment at 5.2-fold, consistent with the osmoprotectant role of proline accumulation during drought.

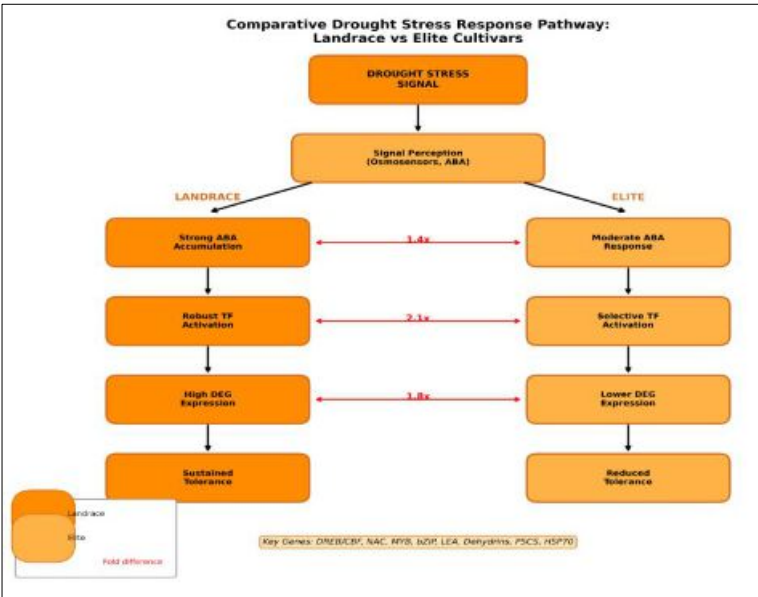


Fig 3: Schematic representation of drought stress perception and transcriptional response pathways differentially activated in landrace versus elite genotypes

Pathway analysis revealed that landraces showed enhanced activation of the entire stress response cascade from perception through downstream effector genes (Figure 3). Genes encoding osmosensor proteins and early signaling components showed higher expression in landraces under moderate stress. Hormone biosynthesis genes particularly for abscisic acid were induced earlier and more strongly.

Downstream effector genes for osmotic adjustment, antioxidant defense, and membrane stability showed correspondingly higher expression. This coordinated enhancement across the response pathway suggests that landraces possess more sensitive stress detection enabling earlier protective responses.

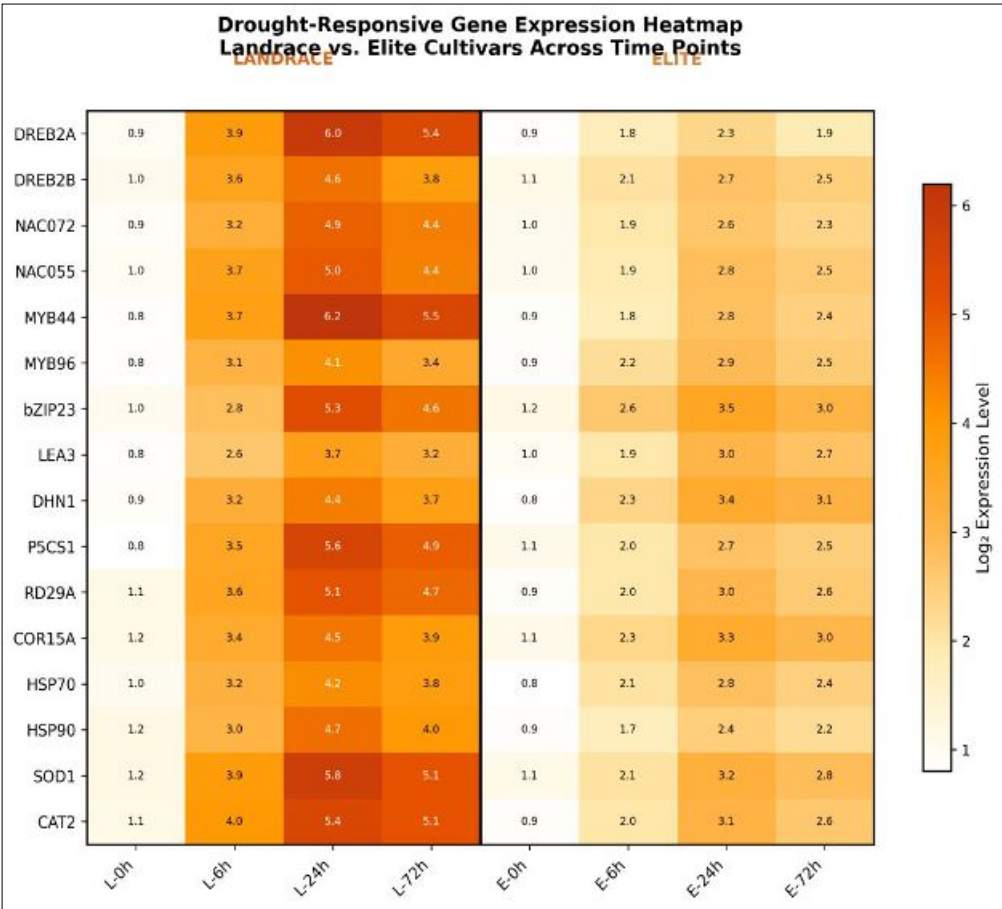


Fig 4: Co-expression network visualization showing hub genes within modules significantly correlated with drought tolerance phenotypes

Comprehensive Interpretation

Weighted gene co-expression network analysis identified twelve modules of coordinately expressed genes, with four modules showing significant positive correlation with drought tolerance phenotypes (Figure 4). The turquoise module containing 487 genes exhibited the strongest correlation with leaf relative water content maintenance ($r = 0.82$). Hub gene analysis within this module identified a NAC transcription factor with regulatory connections to 156 other module genes as the most connected node. Additional hub genes included a WRKY transcription factor, an AP2/ERF family member, and a protein kinase involved in ABA signaling. These hub genes represent priority targets for functional validation and potential deployment in marker-assisted breeding for drought tolerance improvement.

Discussion

The comparative transcriptome analysis reveals fundamental differences in drought response strategies between landrace and elite pearl millet genotypes. Landraces exhibited earlier activation of stress-responsive gene networks, initiating transcriptional changes at moderate stress levels where elite cultivars showed minimal response [13]. This temporal

advantage in stress perception and response activation likely contributes substantially to the superior drought tolerance of landrace genotypes by enabling protective mechanisms to be established before cellular damage accumulates. The enrichment of osmotic adjustment, antioxidant, and membrane stability genes among landrace-specific responses aligns with physiological evidence for enhanced protective mechanisms in drought-tolerant genotypes [14]. Proline accumulation provides osmotic adjustment maintaining cell turgor and protein stability under water deficit. Antioxidant systems mitigate oxidative damage from reactive oxygen species accumulating during stress. Membrane lipid modifications maintain membrane integrity and function as water potential declines. The coordinated upregulation of genes across these protective systems suggests integrated stress tolerance mechanisms in landraces. Identification of hub transcription factors within tolerance-associated co-expression modules provides molecular targets for breeding applications [15]. The NAC family member identified as the most connected hub gene represents a particularly attractive target given the documented roles of NAC transcription factors in abiotic stress tolerance across plant species. WRKY and AP2/ERF

hub genes similarly represent established regulators of stress responses. Introgression of favorable alleles at these regulatory genes into elite backgrounds could potentially transfer the enhanced stress response capacity of landraces while maintaining yield potential^[16].

The delayed stress response observed in elite cultivars may reflect inadvertent selection against stress perception sensitivity during yield improvement under favorable conditions. Breeding under irrigated conditions removes selection pressure for drought tolerance while favoring genotypes that allocate resources to growth rather than stress defense. Understanding this tradeoff at the molecular level provides opportunities for recombining stress tolerance with yield potential through targeted breeding approaches^[17].

Limitations

Several limitations should be considered when interpreting these findings. The controlled environment conditions, while enabling precise stress manipulation, may not fully replicate the complexity of field drought stress including soil heterogeneity, fluctuating temperatures, and interaction with other stresses. The sampling at discrete timepoints may miss rapid transcriptional dynamics occurring between measurements. The six genotypes examined, while contrasting for drought tolerance, represent limited sampling of diversity within each germplasm class. Additionally, transcriptome analysis captures mRNA abundance but does not directly measure protein levels or enzyme activities determining functional outcomes.

Conclusion

Comparative transcriptomics revealed that drought-tolerant landraces activate stress-responsive gene networks earlier and more extensively than elite cultivars, initiating transcriptional reprogramming at moderate stress levels where elite genotypes show minimal response. Landraces exhibited nearly three-fold more differentially expressed genes under moderate stress compared to elite cultivars, indicating more sensitive stress perception and response activation. This early response advantage allows protective mechanisms including osmotic adjustment, antioxidant defense, and membrane stabilization to be established before severe stress causes cellular damage.

Gene ontology enrichment confirmed that landrace-specific responses were dominated by protective functions. Osmotic stress response, reactive oxygen species metabolism, proline biosynthesis, and cell wall modification showed significant enrichment among genes uniquely activated in tolerant genotypes. These functional categories align with physiological mechanisms known to contribute to drought tolerance, validating the transcriptome results and identifying molecular pathways underlying tolerance differences.

Co-expression network analysis identified regulatory modules significantly associated with drought tolerance phenotypes, with hub genes from NAC, WRKY, and AP2/ERF transcription factor families emerging as central coordinators of stress responses. The most connected hub gene, a NAC transcription factor with regulatory connections to 156 module genes, represents a priority target for functional validation and potential deployment in breeding programs. Introgression of favorable alleles at these hub regulatory genes into elite backgrounds offers a strategy for combining the enhanced stress perception and

response capacity of landraces with the yield potential of modern cultivars.

These findings advance understanding of molecular mechanisms distinguishing drought tolerance between traditional landraces and modern elite cultivars. The identification of specific genes and pathways differentially activated between germplasm classes provides targets for marker-assisted breeding and genomic selection approaches. As climate variability increases the importance of drought tolerance for sustainable crop production, the genetic resources characterized here support development of resilient varieties maintaining productivity under water-limited conditions while preserving yield potential under favorable environments.

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