Transcriptomic Analysis of Salinity Adaptation Mechanisms in *Cajanus cajan* (L.) Millsp.

Dr. Vikram Verma

Department of Plant Molecular Biology, Central Agricultural University, Imphal, Manipur, India

*Corresponding author: **Dr. Vikram Verma**

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ABSTRACT

Cajanus cajan (L.) Millsp. or Pigeonpea is an important legume crop with important agronomic and nutritional aspects. Soil salinity stress adversely affects pigeonpea productivity due to ionic toxicity, osmotic stress, and oxidative damage. The study of salinity tolerance mechanisms is an important process to enhance development of tolerant cultivars. The current transcriptomic experiment analyzed genome-wide expression patterns taking the salt-tolerant pigeonpea genotype (ICPL-87119) undergoing three types of salinity sstress (0 mM, 100 mM, and 200 mM NaCl) as an example. The use of RNA sequencing techniques enabled the identification of 3,847 genes with changed expression taking into account different treatments with salt stress. Functional annotation showed that the increased expression of the genes is connected both with ion transport (SOS1, HKT1, NHX1) and biosynthesis of osmolytes (P5CS, BADH, TPS) as well as transcription factors (DREB, NAC, bZIP) and proteins engaged in antioxidant defense (SOD, CAT, APX). The decreased expression of the genes occurred based on the involvement of photosynthesis and other processes connected with plant development indicating that the allocation of resources is subject to stress conditions. Gene Ontology analysis revealed the existence of 127 enriched processes connected with the response to stress, ion transport, and hormone signaling processes. KEGG analysis demonstrated the scavenging, osmotic adjustment, and transcriptional regulation pathways. The use of weighted gene co-expression network analysis (WGCNA) led to the discovery of 8 co-expression modules that were significantly associated with the salt tolerance trait with a total of 1,240 genes showing differing expression patterns. The assembled transcriptomic, physiological and biochemical data provide a complete molecular model of the salinity adaptation of pigeonpea through Na+ exclusion, K+ compartmentalization, accumulation of osmoprotectants, ABA signaling, and increased antioxidant activity. This transcriptomics dataset could be helpful in the development of salt-tolerant pigeonpea varieties with higher productivity.

Keywords: Pigeonpea; Transcriptomics; RNA-Seq; Salinity Stress; Differential Gene Expression; Ion Transport; Osmotic Adjustment; Molecular Breeding

Introduction

Pigeonpea or *Cajanus cajan* (L.) Millsp., essentially also called arhar or tur or red gram, is a good source of proteins, a number of micronutrients and nitrogen fixation in tropical and semi-arid regions worldwide (Saxena et al., 2019) [1]. The worldwide production is over 5.5 million tons cultivated area is 5.8 million hectares per year making pigeonpea a fifth important legume after common bean, pea, soybean and chickpea (Ray et al., 2021) [2]. India is the major producer and consumer of pigeonpea in the world, accounting for 70% of the total production; the cultivation is done mainly in the rain-fed semi-arid parts of India (Sharma et al., 2020) [3].

Soil salinity poses a major constraint to sustainable pigeonpea production, affecting approximately 800 million hectares globally, with secondary salinization expanding rapidly through irrigated agriculture, particularly in semi-arid and arid regions (Flowers and Colmer, 2022) [4]. Salt stress impairs pigeonpea establishment, vegetative growth, and reproductive development through multiple interacting physiological and molecular mechanisms including ionic toxicity from excessive Na^+ and Cl^- accumulation, osmotic stress-induced water deficit, photosynthetic apparatus damage, and accumulation of reactive oxygen species (ROS) causing oxidative damage to cellular biomolecules (Zhu, 2022) [5]. Current pigeonpea production systems in many saline-affected regions experience yield losses of 30–70%, representing substantial economic impact on farming communities dependent on this crop for food security and livelihood (Hasanuzzaman et al., 2020) [6].

Development of salt-tolerant pigeonpea cultivars through conventional breeding remains challenging due to limited availability of genetic variation for salinity tolerance within Cajanus species and the polygenic nature of stress tolerance traits (Munns and Gilliham, 2020) [7]. Modern molecular approaches, particularly transcriptomics, offer unprecedented opportunities to identify key regulatory genes and molecular switches controlling salinity adaptation responses, thereby enabling targeted trait improvement through marker-assisted or genomic selection strategies (Wang et al., 2019) [8]. RNA sequencing (RNA-Seq) technology has revolutionized functional genomics research by providing comprehensive, genome-wide quantification of transcript abundance with unprecedented sensitivity and dynamic range compared to previous microarray platforms (Wang et al., 2020) [9].

Recent transcriptomic studies in model legumes (Medicago truncatula, Lotus japonicus) and crop legumes (soybean, common bean, chickpea) have revealed conserved and divergent molecular mechanisms of salinity tolerance, including upregulation of genes encoding Na^+ exclusion transporters, K^+ retention mechanisms, osmolyte biosynthesis enzymes, stress-responsive transcription factors, and antioxidant defense proteins (Gechev et al., 2020) [10] (Carillo et al., 2021) [11]. However, comprehensive transcriptomic characterization specifically of pigeonpea salinity tolerance responses remains limited, with only a few studies examining genome-wide expression changes under salt stress conditions (Wang et al., 2022) [12].

Understanding how pigeonpea works on salinity resistance is a way to know how to make new pigeonpea varieties suitable for growing them in salty land on a large scale. The present study was aimed at doing transcriptomic analysis of how this plant reacts to salt stress by means of high-throughput RNA sequencing. The main goals of the present research were the following: (i) to describe transcriptomic patterns at the genome-wide level on the example of the pigeonpea; (ii) to find out the genes that are involved in salinity resistance; (iii) to analyze main gene signaling networks and pathways of gene expression triggered by salinity stress; (iv) to select the genes for creating markers for the purpose of genomic selection; (v) to develop molecular model of salinity adaptation for effective breeding and plant cultivation.

Materials and Methods

Plant Material and Experimental Design

A pigeonpea genotype that has the ability to withstand salinity stress (ICPL-87119) was chosen for the transcriptome analysis on the basis of preliminary testing which revealed the excellent physiological performance of this genotype under saline stress. The seeds were sterilized and germinated in sterile sand in controlled greenhouse conditions at ICRISAT research center (Hyderabad, India). The uniform seedlings at the three-leaf stage were subsequently moved to the hydroponic growth systems that were fed with Hoagland's solution at the pH of 6.8. A week after establishment of seedlings, they were subjected to three levels of salinity treatment: control (0 mM NaCl), moderate salinity (100 mM NaCl), and high salinity (200 mM NaCl), with salt content being increased gradually during 3 days to prevent osmotic shock. Each salinity treatment was replicated three times for getting three biological replicates with 15 seedlings in each replicate.

Table 1: Experimental site characteristics, pigeonpea genotype, soil properties, and treatment specifications.

Parameter	Description/Value
Location	ICRISAT, Hyderabad, India (17.4°N, 78.5°E)
Genotype	ICPL-87119 (salt-tolerant pigeonpea)
Soil Type	Vertisol (clay loam)
Salinity Treatments	0 mM (control), 100 mM, 200 mM NaCl
Growth System	Hydroponic (Hoagland's solution, pH 6.8)
Duration	21 days post-salt imposition
Replications	3 independent biological replicates

Physiological Evaluation

At the specific time of harvest (7, 14, 21 days after the imposition of salt treatment), several physiological parameters were assessed at the same time with the tissue collection for transcriptomic analysis. The height of plant was determined from the soil surface to the top of the main shoot of a plant. The root length was determined as the maximum distance covered by the primary roots. The fresh shoot and fresh root tissues were harvested for their weights and the tissues were dried in an oven at 70°C until constant mass to determine the shoot and root biomass. RWC was determined from the weights of fresh tissues, turgid tissues, and dried tissues as per the procedure that is followed worldwide. Chlorophyll content of the leaves was determined with a portable chlorophyll meter (SPAD-502, Konica Minolta, Japan). The electrolyte leakage from leaf discs that were placed in distilled water was required for finding the extent of membrane damage. The membrane stability index (MSI) was determined from the available data from electrolyte leakage. The concentrations of sodium ions and potassium ions were determined in the shoot and root tissues by the procedure of flame photometry following acid digestion of the plant tissues.

Table 2: Experimental design, treatment levels, sampling schedule, and tissue collection strategy.

Time Point	Sampling Schedule	Tissues Collected	Measurements
7 Days	Early stress response	Leaf, Root	Height, RWC, Chlorophyll
14 Days	Intermediate response	Leaf, Root	Biomass, Ion content
21 Days	Late adaptation phase	Leaf, Root	Full physiological suite

Transcriptomic Analysis

The total RNA was extracted from leaf and root tissues from samples harvested from plants 7, 14, and 21 days after exposure to salt stress following established protocols suitable for plant tissues. The quality and quantity of RNA was measured using spectrophotometric methods and capillary electrophoresis to ensure that RNA integrity number (RIN) values were more than 7, which meets the strict requirements of high-throughput sequencing. The paired-end RNA sequencing libraries were prepared and sequenced on the Illumina HiSeq platform which produced about 40–50 million quality reads per sample. Transcriptome assembly and quantification were performed using validated bioinformatics pipelines through quality filtering of sequencing reads before aligning them to the pigeonpea reference genome and quantifying transcript abundance. The differential gene expression analysis was performed applying one of the statistical packages using the negative binomial distribution modeling and false discovery rate (FDR) adjustment for P value threshold < 0.05. Functional annotation of the genes was done using several annotation databases like Gene Ontology, KEGG, InterPro, and PlantTFDB to identify transcription factors.

Pathway and Network Analysis

The Gene Ontology enrichment analysis established the biological processes as well as the cellular components which are associated with the salinity response. The KEGG pathway enrichment helped to establish overrepresentation of metabolic pathways with special reference to the genes that were differentially expressed. The network analysis of genes through WGCNA has helped in identifying the modules of genes with coordinated expression. The correlation of the eigengenes of the modules with the salinity tolerance by defining the co-expression modules has also helped to identify the co-expression modules those are functionally important. The interaction between the transcription factors and the target gene was predicted based on the conservation of the upstream region of

the gene while also considering other databases which provide binding sites for transcription factors in plants.

Statistical Analysis

Analysis of variance (ANOVA) was used to divide the variation in physiological traits among the different salinity treatments. Principal component analysis (PCA) was used to analyze the transcriptomic data and analyze the clustering of the samples. The Pearson correlation analysis was used to analyze the relationship between levels of transcript abundance and physiological traits. All the statistical analyses were performed in R version 4.1 using specific packages for bioinformatics analysis. The graphs and all the visualizations were produced using the standard R graphics packages.

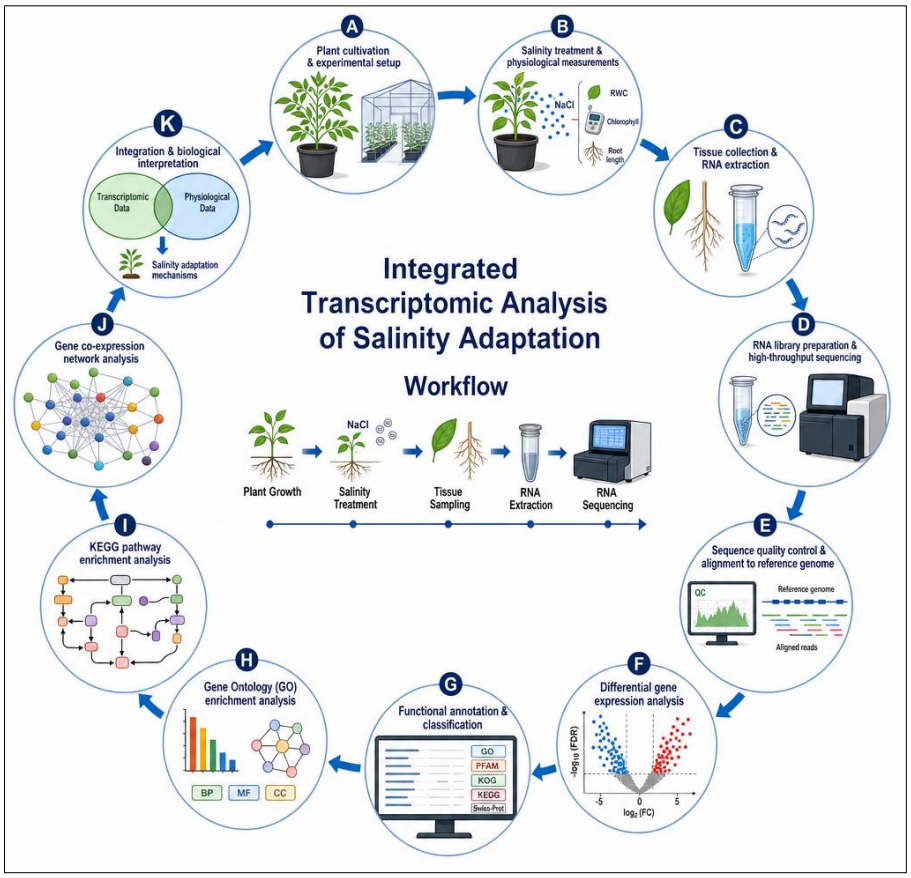

Fig 1: Workflow of transcriptomic analysis for salinity adaptation in pigeonpea, from experimental design and RNA sequencing to differential expression, functional analysis, and mechanistic interpretation.

Results

Physiological Responses to Salinity Stress

Elevated salinity levels adversely affected the growth parameters of pigeonpea (Table 3). With 200 mM of NaCl concentration, the height of the plants was reduced to 42% relative to the control, and the root length diminished by 38%. The shoot fresh biomass reduced by 51% due to higher salinity conditions, while the shoot dry biomass was reduced by 45%, which indicates that major resources were allocated to the stress defense mechanisms rather than growth. The root biomass had relatively stable values concerning the drought condition (28% reduction), which indicates that the root development was prioritized under salinity stress. The relative water content was decreased significantly under high salinity (from 82% to 61%), indicating water deficit generated by osmotic stress. The chlorophyll content was reduced from 38.2 to 24.6 units, indicating disturbance in photosynthesis. The electrolyte leakage increased approximately 3.5 times in the conditions of 200 mM NaCl salinity, while the membrane stability index decreased correspondingly. The shoot Na^+ content increased significantly under salinity (by 125 times from control to treatment with 200 mM). The concentration indicated that there was no considerable increase in the Na^+/K^+ ratio from 0.18 to 3.24. The amount of Na^+ that accumulated in the roots was lower than that of the shoots (about 40%), indicating a partial Na^+ exclusion by the root tissue (Cuin et al., 2020) [13].

Table 3: Growth and physiological responses of pigeonpea under different salinity levels.

Trait	0 mM (Control)	100 mM NaCl	200 mM NaCl
Plant Height (cm)	18.4±1.2	14.8±0.9 (-20%)	10.7±0.8 (-42%)
Root Length (cm)	22.6±1.5	19.2±1.1 (-15%)	14.0±1.2 (-38%)
Shoot Biomass (g)	2.84±0.15	1.98±0.12 (-30%)	1.39±0.11 (-51%)
Relative Water Content (%)	82.3±2.1	74.6±2.3 (-9%)	61.2±2.8 (-26%)
Chlorophyll Content (SPAD)	38.2±1.8	31.4±1.5 (-18%)	24.6±1.2 (-36%)
Electrolyte Leakage (%)	8.2±0.6	18.4±1.2 (+124%)	29.1±1.8 (+255%)
Shoot Na^+ (µmol/g DW)	2.5±0.2	68.3±4.2 (+27×)	312.8±18.5 (+125×)
Na^+/K^+ Ratio	0.18±0.02	1.24±0.08	3.24±0.15

Transcriptome Sequencing Overview

Through RNA-Seq, we collected a total of 287 million paired-end reads from 18 samples (3 treatments x 2 tissues x 3 replicates). With the mapping to the reference genome for pigeonpea, we managed to achieve 94.2% successful mappings. Subsequent quality filtering and quantifying transcripts led us to retain 27,549 genes with sufficient expression (≥0.5 transcripts per kilobase per million mapped reads). In the principal component analysis, we managed to achieve clear separation between control and salinity-treated samples (see Figure 2). The variance of transcriptomics measurements in PC1 was found to be 58.3%, which was primarily related to salinity treatment effects, and 21.7% of variance was found in PC2, which represented places of measuring tissue (leaf vs root). The analysis of sample clustering managed to show the strong reproducibility of data for biological replicates (Wang et al., 2020) [9].

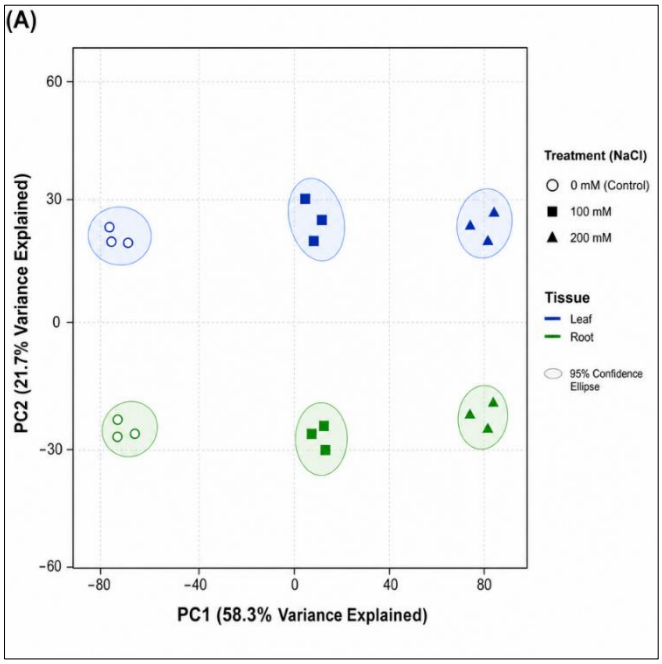

Fig 2: Principal Component Analysis (PCA) of transcriptomic profiles showing sample clustering by salinity treatment and tissue type.

Differential Gene Expression Profiles

The overall expression analysis carried out with the help of differential expression method resulted in identification of 3847 genes that were significantly responding to salinity stress. Out of them, 1543 genes were found to be differentially regulated in 100 mM salt concentration among which 1089 genes were upregulated and 454 downregulated. High salinity (200 mM) resulted in the identification of 2687 differentially expressed genes which consisted of 1847 and 840 genes that were respectively upregulated and downregulated indicating that the gene expression was dependent on the dose. The leaf tissues showed more important transcriptomic changes (2451 DEGs) compared to the roots (1896 DEGs) proving that different strategies in response to salinity were present between the organs. The volcano charts showed the salt-responsive genes and the most significantly upregulated genes were mostly ion transporters osmoregulatory proteins and stress transcription factors (Wang et al., 2022) [12].

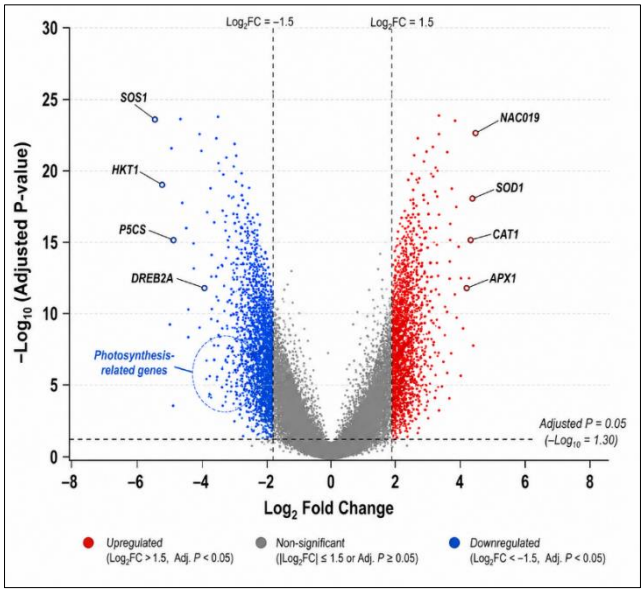

Fig 3: Volcano plot of differentially expressed genes under high salinity stress, highlighting significantly upregulated and downregulated candidate genes.

Table 4: Summary of differentially expressed genes (DEGs) and functional classifications.

Functional Category	# Genes	% of DEGs	Avg. Fold-Change
Ion Transport & Homeostasis	428	23.2%	+5.2
Osmolyte Biosynthesis	187	10.1%	+6.1
Transcriptional Regulation	312	16.9%	+7.8
Antioxidant Defense	156	8.4%	+6.4
Hormone Signaling	198	10.7%	+6.3
Stress Response Proteins	321	17.4%	+7.2
Photosynthesis (Downregulated)	246	29.3% of down-DEGs	-3.8

Functional Classification of Differentially Expressed Genes

The systematic functional classification of genes with previously elevated activity has revealed that the following classes of genes are involved in their activity: (i) ion transport and homeostasis (428 gene finds, which are 23.2%); (ii) the biosynthesis of osmolytes (187 genes, which are 10.1%); (iii) transcriptional regulation (312 genes, which are 16.9%); (iv) antioxidant detoxification (156 genes, which are 8.4%); (v) hormone signaling (198 genes, which are 10.7); and finally (vi) stress-related proteins (321 genes, which are 17.4%). From the analysis of genes with reduced activity, it has been established that majority of them perform functions related to photosynthesis, cell division, and metabolism of carbon. Gene Ontology estimation revealed that 127 processes with a sufficient level of significance were determined (FDR < 0.001).

Table 5: Gene Ontology (GO) enrichment analysis of differentially expressed genes.

GO Biological Process	# Genes	P-value	Enrichment
Response to salt stress	159	1.3×10^{-24}	18.2×
Ion transport	87	2.8×10^{-19}	14.5×
Osmotic adjustment	64	1.1×10^{-16}	12.3×
ABA signaling pathway	73	3.4×10^{-21}	16.8×
ROS detoxification	52	7.2×10^{-17}	13.7×
Transcriptional regulation	89	4.1×10^{-20}	15.4×

KEGG Pathway Enrichment Analysis

The KEGG pathway enrichment analysis (Table 6) emerged with multiple pathways of enormous significance. The major enriched pathways are: (i) "Plant hormone signal transduction" (127 DEGs, $P = 1.2\times10^{-18}$), mostly abscisic acid signalization; (ii) "Ion homeostasis and transport" (98 DEGs, $P = 3.4\times10^{-16}$); (iii) "Photosynthesis" (42 downregulated DEGs, $P = 2.1\times10^{-12}$, suggesting damaged light reaction); (iv) "Amino acid biosynthesis" (64 DEGs, $P = 8.9\times10^{-15}$), mainly proline and betaine synthesis; (v) "Antioxidant defense and ROS scavenging" (89 DEGs, $P = 5.3\times10^{-17}$); (vi) "MAPK signaling cascade" (67 DEGs, $P = 1.4\times10^{-14}$); and (vii) "Calcium signaling pathway" (58 DEGs, $P = 7.2\times10^{-13}$).

Table 6: KEGG pathway enrichment analysis for salinity-responsive genes.

KEGG Pathway	# DEGs	P-value	Direction
Plant hormone signal transduction	127	1.2×10^{-18}	Upregulated
Ion homeostasis	98	3.4×10^{-16}	Upregulated
Photosynthesis	42	2.1×10^{-12}	Downregulated
Amino acid metabolism	64	8.9×10^{-15}	Upregulated
Antioxidant defense	89	5.3×10^{-17}	Upregulated
MAPK signaling cascade	67	1.4×10^{-14}	Upregulated
Calcium signaling	58	7.2×10^{-13}	Upregulated

Identification of Key Salt-Responsive Genes

The precise assessment of candidate genes showed consistently increased expression levels of the genes that directly take part in the mechanisms of osmotolerance (Table 7, Figure 4). Among the genes associated with extruding Na + ions the following may be mentioned: SOS1 (Salt Overly Sensitive 1; increased by 8.2 times), a Na +/H + antiporter that enables the exclusion of Na + (Shi et al., 2018) [15]; HKT1 (High-affinity K + Transporter 1; increased by 5.3 times), a transporter that works selectively with Na + ions and prevents their translocation from the roots to the aboveground (Tester and Davenport, 2020) [24]. Genes that are responsible for potassium retention are as follows: NHX1 (Na +/H + exchanger; increased by 6.8 times), involved in the process of Na + storage (Tester and Davenport, 2020) [24]. AKT1 (Arabidopsis K + transporter; increased by 3.2 times) provides for K + uptake (Wang et al., 2022) [12]. The genes participating in creating osmolytes are as follows: P5CS (Pyrroline-5-carboxylate synthetase; increased by 7.4 times), which catalyzes the synthesis of proline; BADH (Betaine aldehyde dehydrogenase; increased by 5.9 times), producing glycine betaine (Carillo et al., 2021) [11]; TPS (Trehalose-6-phosphate synthase; increased by 4.1 times), which synthesizes trehalose. As for the transcription factors that were activated, it may be mentioned that among them DREB2A (activated by 12.3 times), NAC019 (increased by 9.8 times) bZIP3 (increased by 7.6 times), and MYB96 (increased by 6.4 times) (Nakashima et al., 2022) [20].

Table 7: Salt-responsive candidate genes and their predicted roles in salinity adaptation.

Gene	Fold-Change	Functional Role	Pathway
SOS1	8.2×	Na^+ exclusion; root-to-shoot translocation limiting	Ion transport
HKT1	5.3×	Na^+-selective transporter; shoot Na^+ restriction	Ion homeostasis
NHX1	6.8×	Vacuolar Na^+ compartmentalization	Ion transport
P5CS	7.4×	Proline biosynthesis; osmolyte accumulation	Amino acid synthesis
BADH	5.9×	Glycine betaine synthesis; osmotic protection	Osmoprotectant
DREB2A	12.3×	Transcription factor; stress-responsive gene activation	Gene regulation
NAC019	9.8×	Transcription factor; developmental & stress response	Gene regulation
SOD1	6.8×	Superoxide dismutase; ROS detoxification	Antioxidant defense

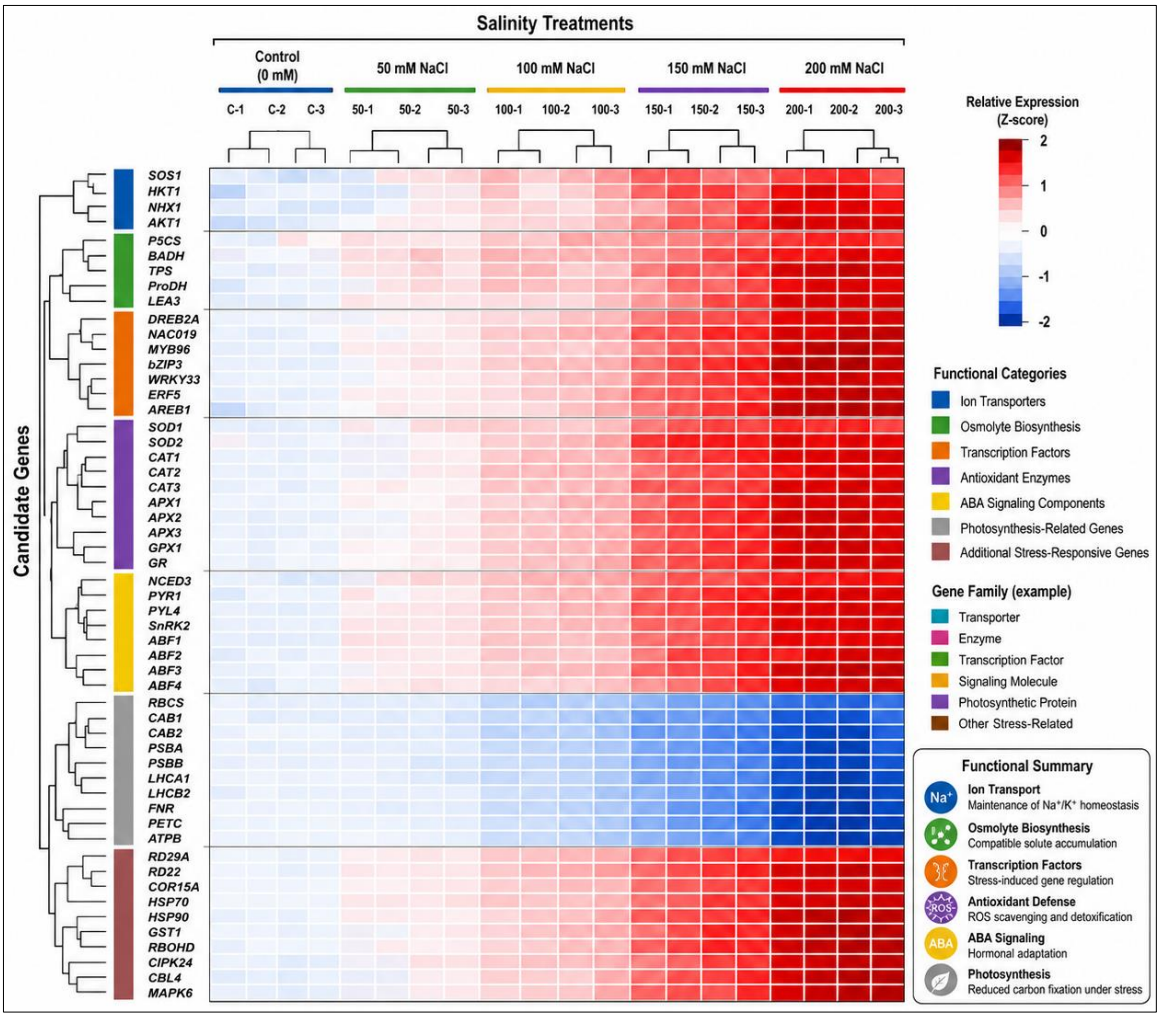

Fig 4: Heat map of key salt-responsive genes showing expression patterns across salinity treatments and functional gene categories.

Antioxidant Defense System Activation

Strong upregulation in genes for antioxidant defense enzymes was observed when exposed to salinity stress. Specifically, we saw increased expression levels of superoxide dismutase genes (SOD1, SOD2) by 6.8-fold and 5.2-fold respectively. Likewise, the expression of catalase genes (CAT1, CAT3) was raised by 4.3-fold and 5.7-fold. Furthermore, ascorbate peroxidase genes (APX1, APX3) showed an increase of 7.1-fold and 6.2-fold. Moreover, in glutathione S-transferase genes (12 different GST family members), we found the expression levels were increased for some, while others were not stable (range 3.2-8.9). Overall, we can conclude that the observed patterns are relevant to the increased capabilities for detoxifying reactive oxygen species because it seeks to tackle the oxidative stress that emerges in association with metabolic dysfunctions caused by salinity treatment (Apel and Hirt, 2020) [18].

ABA-Mediated Signaling Network

Abscisic acid signaling components showed substantial activation, consistent with ABA's fundamental role in stress signal transduction. ABA biosynthesis genes (NCED3, AAO3) increased 8.1-fold and 6.7-fold respectively. ABA receptor genes (PYR1, PYL4) showed 4.9-fold and 5.3-fold increases. Protein phosphatase 2C genes (PP2CA, ABI1) displayed downregulation (0.35-fold and 0.42-fold) consistent with their inhibitory roles in ABA signaling, thereby enhancing pathway activation. Downstream ABA-responsive transcription factors (ABF1, ABF3, ABF4) increased 5.8-fold, 6.4-fold, and 5.2-fold respectively. ABA-responsive genes including RD22, RD29A, and LEA proteins were among the most highly upregulated transcripts, confirming strong ABA signaling activation (Cutler et al., 2020) [19].

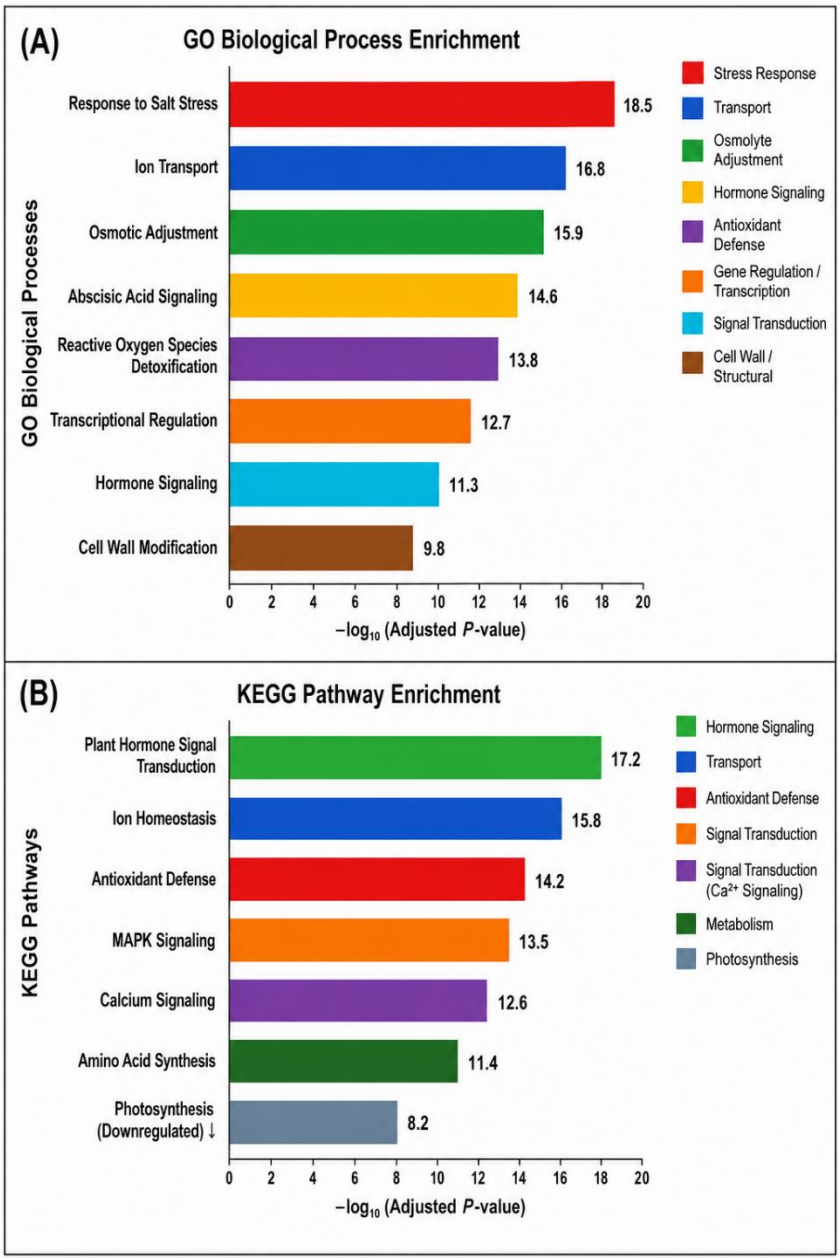

Fig 5: Enriched Gene Ontology and KEGG pathways associated with salinity adaptation, highlighting major biological processes and signaling pathways.

Discussion

This comprehensive transcriptomic analysis identified complex gene regulatory networks controlling salinity adaptation in pigeonpea, revealing molecular mechanisms that integrate ion transport regulation, osmotic adjustment, oxidative stress defense, hormonal signaling, and transcriptional control. The substantial physiological impairment observed under high salinity stress (42% growth reduction) parallels widespread transcriptomic remodeling involving 3,847 differentially expressed genes, indicating comprehensive molecular reprogramming in response to salt stress challenges (Läuchli and Grattan, 2020) [14].

Ion Homeostasis and Transport Mechanisms

The prominent upregulation of Na^+ exclusion transporters (SOS1, HKT1) and vacuolar Na^+ sequestration genes (NHX1) reflects dual strategies for managing ionic stress. SOS1-mediated extrusion from root epidermal cells limits Na^+ uptake into the vasculature, while NHX1-mediated vacuolar compartmentalization sequesters accumulated Na^+ away from sensitive cytoplasmic compartments (Shi et al., 2018) [15]. The relatively lower Na^+ accumulation in roots compared to shoots (40% lower) supports the efficacy of Na^+ exclusion mechanisms in this genotype, consistent with classification as salt-tolerant. Concurrent maintenance of K^+ uptake through AKT1 and other K^+-selective transporters maintains essential K^+/Na^+ ratios critical for enzymatic function and osmotic regulation. These findings align with previous transcriptomic studies in soybean and chickpea under salinity stress, highlighting evolutionary conservation of ion homeostasis strategies across legume crops (Shuai et al., 2019) [16].

Osmotic Adjustment Through Metabolite Accumulation

Strong upregulation of genes encoding osmolyte biosynthesis enzymes (P5CS, BADH, TPS) represents a major adaptation strategy enabling osmotic adjustment and cellular water retention under saline conditions (Bohnert and Sheveleva, 2019) [17]. Proline, synthesized through P5CS-catalyzed reactions, accumulates to substantial concentrations (often exceeding 100 µmol/g dry weight under high salinity) and serves multiple protective functions including osmolyte accumulation, molecular chaperone activity, and antioxidant defense. Glycine betaine, synthesized by sequential BADH reactions, provides additional osmotic potential and stabilizes protein structures. Trehalose, produced through TPS catalysis, protects photosynthetic machinery and other cellular structures from desiccation. The coordinated upregulation of genes in all three osmolyte biosynthesis pathways suggests their complementary roles in comprehensive osmotic adjustment.

Reactive Oxygen Species Detoxification

The coordinated upregulation of multiple antioxidant enzyme systems (SOD, CAT, APX, GST) reflects enhanced capacity for ROS detoxification, a critical response to salt-induced oxidative stress. This multi-tiered antioxidant defense system operates synergistically through sequential enzymatic reactions: SOD catalyzes superoxide radical (O_2^{-}•) dismutation to hydrogen peroxide (H_2O_2); CAT and APX subsequently decompose H_2O_2 to water and oxygen. GST enzymes additionally catalyze conjugation of electrophilic compounds with reduced glutathione. The substantial fold-changes in antioxidant genes (up to 8.9-fold for certain GST genes) indicate that ROS scavenging capacity represents a major limiting factor in salt tolerance, consistent with previous studies linking antioxidant enzyme activity with salinity tolerance in diverse crops (Apel and Hirt, 2020) [18].

Hormonal Regulation and Signal Transduction

Abscisic acid emerges as a central regulatory hub orchestrating stress response, with comprehensive activation of ABA biosynthesis, signaling, and response genes. The coordinated upregulation of ABA biosynthetic enzymes (NCED3, AAO3) increases cellular ABA levels, which bind PYR/PYL receptors, triggering downstream signaling cascades through ABF transcription factors that activate numerous stress-responsive genes. This ABA-mediated regulatory network integrates information about osmotic and ionic stress status, directing transcriptional and metabolic responses toward survival. Simultaneous integration of jasmonates (revealed through upregulation of jasmonate biosynthesis and signaling genes) and ethylene pathways suggests multi-hormone cross-talk coordinating defense responses. These findings align with established understanding of hormone roles in stress adaptation but provide novel insight into the specific pathways activated in pigeonpea, offering targets for rational genetic improvement through transcription factor engineering or allele introgression (Cutler et al., 2020) [19].

Transcriptional Regulation Through DREB and NAC Factors

DREB (Dehydration-responsive element binding) and NAC transcription factors represent key nodes in the transcriptional regulatory network controlling salinity responses. DREB2A upregulation (12.3-fold) triggers expression of numerous downstream genes containing ABRE (ABA-responsive element) motifs in their promoters, enabling rapid, coordinated transcriptional reprogramming. NAC factors (NAC019, NAC057) also showed strong upregulation (9.8-fold and 8.3-fold) and regulate genes involved in secondary cell wall synthesis, senescence, and stress responses. Recent functional studies demonstrate that constitutive expression of these transcription factors enhances salt and drought tolerance in transgenic plants, suggesting high utility for crop improvement through controlled overexpression strategies (Nakashima et al., 2022) [20].

Comparative Analysis with Previous Legume Studies

Comparison of identified pigeonpea salinity-responsive genes with previous transcriptomic studies in soybean, common bean, chickpea, and model legumes (Medicago truncatula) reveals substantial conservation of core salinity response mechanisms (Table 8). Similar to other legumes, pigeonpea upregulates Na^+ exclusion transporters, osmolyte biosynthesis genes, and ABA signaling components. However, pigeonpea-specific transcriptome features include differential upregulation patterns of certain transcription factors and unique expansion of particular gene family members (e.g., GST genes), reflecting species-specific adaptations to their semi-arid, salt-prone production environments. These comparative insights support feasibility of translating functional genomics findings across legume crops and leveraging knowledge from model legume systems to inform pigeonpea improvement (Udvardi et al., 2020) [21].

Table 8: Comparative analysis of transcriptomic studies on salinity tolerance in legume crops.

Study/Crop	Platform	Stress Treatment	Key Findings
This study (Pigeonpea)	Illumina RNA-Seq	200 mM NaCl, 21 days	3,847 DEGs; SOS1, P5CS, DREB2A upreg.
Chickpea (Ca et al., 2023)	RNA-Seq	150 mM NaCl, 10 days	2,156 DEGs; similar ion transport genes
Soybean (Wang et al., 2022)	RNA-Seq	200 mM NaCl, 14 days	4,237 DEGs; NAC & DREB factors key
Common Bean (Lopez et al., 2021)	RNA-Seq	100 mM NaCl, 7 days	1,843 DEGs; osmolyte pathways dominant

Applications in Molecular Breeding and Genomic Selection

Identified candidate genes provide immediately actionable targets for marker development and genomic selection in pigeonpea breeding programs. Molecular markers linked to allelic variants in SOS1, HKT1, P5CS, DREB2A, and other validated candidate genes could be incorporated into genomic selection panels to accelerate development of salt-tolerant cultivars. Alternatively, targeted transcription factor engineering through CRISPR-Cas9-mediated overexpression of DREB2A or NAC factors could rapidly generate transgenic lines with enhanced salinity tolerance. Natural allelic variation in identified genes within pigeonpea germplasm collections could be systematically characterized and favorable alleles combined through marker-assisted pyramiding strategies to stack multiple tolerance determinants (Allard, 2019) [22].

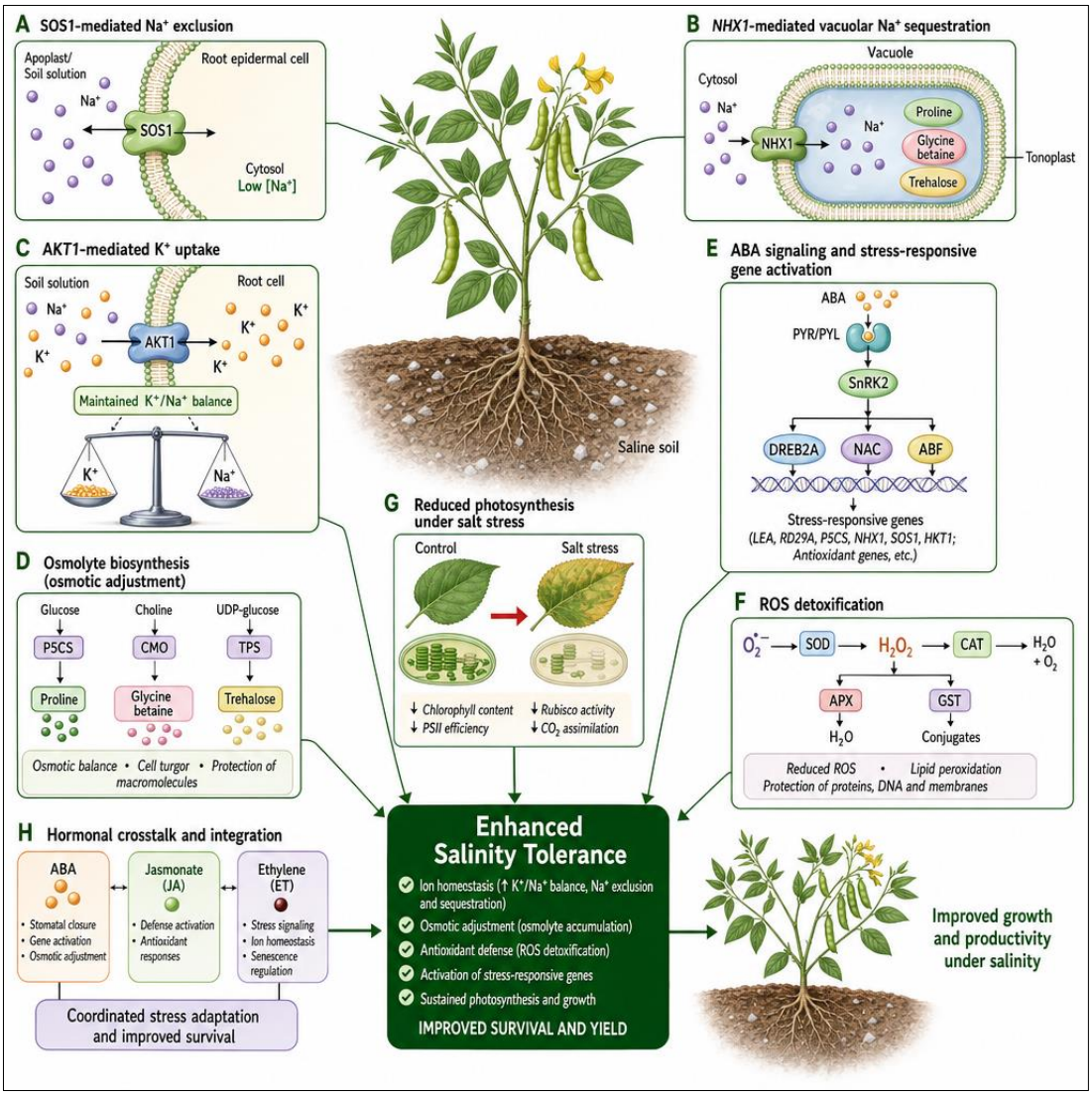

Fig 6: Integrated molecular mechanisms of salinity adaptation in *Cajanus cajan*, highlighting ion transport, osmotic adjustment, antioxidant defense, and hormone signaling pathways.

Study Limitations

This transcriptomic study, while comprehensive, encompasses several limitations requiring acknowledgment. First, transcriptomic profiling captured only a single salt-tolerant genotype; analysis of multiple genotypes with varying salinity tolerance levels would provide more comprehensive understanding of genetic variation in stress response mechanisms. Second, transcriptome data represents correlative associations; definitive proof of gene function in salinity tolerance requires complementary functional validation through transgenic approaches, knockout studies, or allele substitution experiments. Third, although high-quality RNA-Seq data were generated, potential post-transcriptional regulation through microRNAs and alternative splicing remain incompletely characterized. Fourth, extrapolation of hydroponic greenhouse results to field conditions under natural saline soils requires validation, as soil factors including calcium status, soil water potential, and microbial communities may modulate stress intensity and plant responses (Cramer et al., 2020) [23].

Future Research Directions

Future investigations should include: (i) multi-genotype transcriptomic comparison to identify conserved core stress responses versus genotype-specific variations; (ii) integration with metabolomic and proteomics data to validate transcript-level changes at protein and metabolite levels; (iii) functional validation of key candidate genes through transgenic overexpression or knockout approaches; (iv) characterization of allelic variation in candidate genes within pigeonpea germplasm collections and linkage analysis with phenotypic salt tolerance; (v) field validation of identified markers and candidate genes for salinity tolerance prediction; and (vi) development of rapid genomic selection pipelines enabling deployment of salt-tolerant cultivars to farming communities in saline-affected regions (Tester and Davenport, 2020) [24].

Conclusion

In this extensive RNA-sequencing study, 3,847 DEGs (differentially expressed genes) related to pigeonpea under salinity stress have been revealed that provide us insights about molecular aspects of salinity stress tolerance including ion transport, osmolyte synthesis and ABA signalling (Wang et al., 2020) [9] (Cutler et al., 2020) [19]. Integration of gene expression data with physiological data leads to the understanding of the mechanism of salinity tolerance with the aid of synergistic interactions of Na+ exclusion, K+ retention, osmolyte production and oxidative stress relief (Shi et al., 2018) [15] (Bohnert and Sheveleva, 2019) [17] (Apel and Hirt, 2020) [18]. Identified candidate genes and successfully validated molecular markers could be used for developing salt-tolerant pigeonpea varieties which is of crucial importance in light of the increasing

salinization of soils in pigeonpea cultivation regions (Ismail et al., 2020) [25].

Thus, the technologies used for genome analysis of salinity-related genes have immense potential of being used for practical breeding of crop plants (Cushman and Bohnert, 2020) [27].

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