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RESEARCH ARTICLE

Development and validation of candidate gene specific markers for salinity responsiveness in chickpea (*Cicer arietinum* L.)

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Abstract

Soil salinity is a crucial abiotic stress that significantly reduces the yield and productivity of crops, it reduces chickpea annual yields by 8 to 10% globally. The efficient utilization of marker-aided selection would increase precision and expedite the development of salt-tolerant chickpea varieties. This study intends to identify and analyze microsatellite regions from identified candidate genes related to salinity in chickpeas. A total of 195 putative candidate genes were sort listed, out of which 158 genes contained microsatellite loci with mono- (32%) and di- (32%) nucleotides, followed by tri- (18%), tetra- (11%), penta- (4%) and hexa-nucleotides (3%) motifs for salt tolerance. Gene-based SSR (cg-SSR) primer sets were used to validate the results in a panel of tolerant and sensitive genotypes. The polymorphic cg-SSR loci gave a polymorphic information content (PIC) ranging from 0.21 to 0.37 with an average of 0.34. A neighbor-joining (N-J) tree was created to depict the relationships between genotypes, classifying them into two distinct clusters. The first group was most salt-sensitive, while the second mainly contained salt-tolerant genotypes, which were congruent with the phenotypic data. The analysis of population structure unveiled the presence of two sub-populations. Sub-population 1 consisted of salt-tolerant genotypes, while sub-population 2 comprised salt-sensitive genotypes. The study ultimately revealed that the Cg-SSR markers designed for salinity were effective in deciphering the functional diversity and salinity responses in chickpeas.

Keywords: Chickpea, Cg-SSRs, genetic diversity, microsatellite, salinity.

Introduction

Chickpea (*Cicer arietinum* L.), is an important grain legume with a genome size of ~740 Mb (Varshney et al. 2013). The crop has seen a significant increase in production from 9.38 million tons (2016–17) to 11.53 million tons (2024–25) (MoA & FW GOI, 2025). Being rich in its nutritional profile 50 to 58% carbohydrates, 15 to 22% protein, 3.8 to 10.2% fat, and essential micronutrients (<1%) makes it globally more significant (Jukanti et al. 2012). However, increasing climatic disturbances have heightened biotic and abiotic stresses, especially salinity, which severely impact chickpea growth, development, and yields (Kumar et al. 2020).

Further breeding for salinity is limited by the complex nature of the trait, which is highly influenced by environmental factors. To overcome cumbersome and error-prone phenotyping for salinity, it is best to adapt molecular markers to increase the overall efficiency, precision and accuracy (Elshafei et al. 2019) of any salinity breeding program. Microsatellite markers, referred to as SSRs (simple sequence repeats), are a preferred choice for molecular breeding due to their co-dominant characteristics, excellent repeatability, multiple allelic variations, high transferability, and low-cost analysis. Traditionally, markers

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obtained from random genomic sequences may have an uncertain linkage to the transcribed regions (genes) of the genome, which is the major limitation. As a modification, microsatellite markers obtained from potential candidate genes may greatly hasten the marker-aided breeding program for the targeted trait (Hasan et al. 2021). The most efficient technique for determining these genic markers is transcriptome profiling (RNA-Seq), which has tremendous potential for locating SSRs within the transcribed genome region. Genic SSRs derived from the genome's coding region have demonstrated strong cross-transferability to related taxa, consistent amplification, and resilience, as well as relationships with specific traits (Mehta et al. 2021). Genic SSRs have played a crucial role in comparative genome mapping and evolutionary relationship analysis, as they can be easily transferred for studies in other related species (Meena et al. 2024). The genomic regions associated with responsive genes along with the yield traits have also been mapped in chickpea RILs (recombinant inbred lines) derived from ICCV 10 (salt tolerant) and DCP92-3 (salt sensitive) crosses of population size of 201 (Soren et al. 2020). The same set of parents of the mapping population (ICCV 10 and DCP 92-3) along with two cheeks JG 11(salt tolerant) and Pusa 256 (salt sensitive) were also used for a comprehensive understanding of transcriptome data via integrating physiological analysis for better understanding of molecular mechanics for salt tolerance in Chickpea (Kumar et al. 2021). The combination of these two studies has established a foundation for identifying potential candidate genes for salinity responsiveness in the present investigation. Specific genes associated with abiotic stress tolerance have been implicated in conferring salt tolerance and encompass a wide array of protein classes, including vacuolar Na^+/H^+ antiporters (V-H^+-ATPase and V-H^+-pyrophosphatase), plasma membrane Na^+/H^+ transporters, high-affinity K transporters (HKT), transcription factors (MYB, WRKY, DREB etc.), aquaporins (AQP), signaling proteins and kinases, as well as antioxidants (Kumar et al. 2021). Developing gene-based SSR markers from these salt-responsive candidate genes would prove immensely beneficial. Nevertheless, scant supporting evidence exists regarding the development of genic SSR markers derived from candidate gene sequences (genic SSRs), as indicated by the studies conducted by Mehta et al. (2021) and Molla et al. (2015). The current study utilizes 195 candidate genes in chickpea, verified via forward and reverse genetics for SSR marker analysis (Soren et al. 2020; Kumar et al. 2021), and aims to identify, mine, and validate these novel salt-responsive genic SSR (cg-SSR) markers in chickpea.

Materials and methods

Identification of candidate genes responsive to salt stress

A detailed analysis of genes within QTL regions and RNA-seq data (Soren et al. 2020; Kumar et al. 2021) identified 1121 genes based on gene ontology (GO) annotation and literature survey. Of these, 195 candidate genes, mainly located on linkage groups CaLG03 and CaLG06, were shortlisted (Soren et al. 2020). Supplementary transcriptome profiling in similar genotypes further identified differentially expressed genes under salt stress (Kumar et al. 2021), highlighting 40 common candidate genes, primarily in CaLG03 and CaLG06, which play crucial roles in cellular integrity, stress perception, signaling, and transport functions. Candidate genes within the QTL regions were identified through BLAST searches against the chickpea reference genome using flanking markers. To confirm gene roles and differential expression, RNA-seq data were generated from root samples of chickpea genotypes under normal and stress conditions (*ICCV 10, JG 11, DCP 92-3, Pusa 256*) (Kumar et al. 2021). These RNA-seq datasets, published in NCBI's SRA under BioProject ID: PRJNA579008, were used for mining genes associated with salinity tolerance.

SSRs mining and primer designing

The identified candidate genes conferring salt tolerance in chickpeas were subjected to mining SSRs from their FASTA sequences. SSRs were mined using the Krait tool, which contained the complete gene sequence, encompassing untranslated regions, promoter regions, intron and exons (Du et al. 2018). Also, we analyze the occurrence and distribution of SSRs (single nucleotide repeats, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, pentanucleotide repeats, and hexanucleotide repeats) within the candidate gene sequence. Default settings of the Krait tool, defining repeat prevalence at values of 10, 7, 5, 4, 4, and 4 for mono-, di-, tri-, tetra-, penta-, and hexanucleotide repeats, respectively, were employed to predict SSRs. Subsequently, primers for PCR amplification and validation were designed using Primer 3 software, utilizing flanking sequences identified from the microsatellite repeat region.

Plant materials and screening for salinity

This study examined 24 chickpea genotypes with varied responses to salt stress (Table 1). Phenotyping was conducted at ICAR-Indian Agricultural Research Institute, New Delhi, under controlled environmental conditions (22/18 °C day/night, 10/14 h light/dark, 45 ± 5% relative humidity) using PVC pots (18x35 cm, 7.5 L). Each pot contained 7.0 kg of soil (initial EC = 0.4 dS/m) and was fertilized with 2 g of di-ammonium phosphate (DAP). Salt stress was induced a week before sowing by applying a 60 mM NaCl solution (1.50 L per pot) to reach field capacity. After sowing, pots were watered with reverse osmosis (RO) water to maintain field capacity and prevent excess salt accumulation. Weekly EC monitoring ensured a consistent 6 ± 0.2 dS/m level. Control pots received only RO water, with no NaCl treatment. Each pot was initially sown with five seeds, and later, after two weeks, the seedlings were thinned to two. Three replicates

for control and treatment groups were maintained, and analyses were conducted using a completely randomized design.

Phenotyping under salt stress and data analysis

Chickpea genotypes were evaluated for yield traits such as days to 50% flowering (DOF), days to maturity (DOM), plant height (PH), number of pods per plant (NPPP), seeds per five pods, plant yield (SY), and 100-seed weight (100 SW). Data from three replications were recorded for control and stress treatments. Physiological assessments included relative water content (RWC) and membrane stability index (MSI), following Barrs and Weatherley (1962) and Blum and Ebercon (1981). Salt tolerance indices (STI) were determined using the formula by Joshi et al. (2023). Statistical analyses, including coefficient of variance (CV), critical difference (CD), analysis of variance (ANOVA), and standard deviations (SD), were conducted using R programming. Genotypes served as the main factor, while salinity was treated as a sub-factor in a two-factor Completely Randomized Design (CRD) for data evaluation using the R statistical environment (R 4.2.1; R Core Team 2024).

DNA Extraction and Marker Amplification

DNA was extracted from the leaf tissues using the CTAB method (Murray and Thompson, 1980) and quantified using a spectrophotometer. The quality was assessed on a 0.8% agarose gel. Twenty cg-SSR markers were selected for validation among 24 diverse chickpea genotypes. Each reaction mixture contained the following components for PCR amplification: 1-µL of 20 ng plant genomic DNA, 1.6 µL of 10 x Tris buffer having 1.5 mM $MgCl_2$, 1-µL of 10 mM dNTP mix, 1.0 µl each of forward and reverse primers, and 0.3 µL of Taq polymerase (3 U/µL). The steps followed by the thermal cycler were as follows: i) DNA denaturation at 94°C for 3 minutes, ii) 40 cycles of 94°C for 30 seconds, iii) 50–57°C for 1 minute, and iv) 72°C for 10 minutes. The amplicons were analyzed on 3% agarose gels (Cambrex, USA) stained with ethidium bromide and visualized using gel documentation unit (Alphalmager 2200, Alpha Innotech Corp., USA).

Table 1. List of 24 Chickpea genotypes used for phenotyping and validation of cg-SSR markers under elevated salinity

S. No.	Genotype name	Type	Pedigree	Country of Origin
1	CSG8962	*Desi*	Selection from GPF 7035	CSSRI, Karnal, India
2	JG62	*Desi*	Local selection from west Nimar (M.P.)	JNKVV, Jabalpur, India
3	PUSA372	*Desi*	P1231 x P1265	IARI, New Delhi, India
4	ICCV06101	*Desi*	ICC 5619 x ICC 37	ICRISAT, India
5	ICCV00104	*Desi*	JG 74 x ICCL 83105	ICRISAT, India
6	ICCV10111	*Desi*	ICCV 93954 x ICC5683	ICRISAT, India
7	ICCV10	*Desi*	P1231 × P1265	ICRISAT, India
8	JG11	*Desi*	([PhuleG5 x Narshinghpur bold]xICCC37)	JNKVV, Jabalpur, India
9	PUSA1103	*Desi*	(BG 256 x *Cicer reticulatum*) x BG 362	IARI, New Delhi, India
10	AVARODHI	*Desi*	(T-3 x K 315)	CSAU&T, Kanpur, India
11	BGD112	*Desi*	(BG 209 x GL 84038) x Pusa 212	IARI, New Delhi, India
12	ANNEGIRI	*Desi*	Local selection from germplasm of Karnataka	UAS, Dharwad, India
13	ICC4958	*Desi*	Genetic stock, collected from Jabalpur, Madhya Pradesh	JNKVV, Jabalpur, India
14	PUSA547	*Desi*	Mutant of BG 256	IARI, New Delhi, India
15	SBD377	*Desi*	(ICCV 88109 x PRR 1) x ICC 4958	IARI, New Delhi, India
16	PUSA362	*Desi*	(BG 203 x P 179) x BC 203	IARI, New Delhi, India
17	ICCV2	*Kabuli*	[(K850 x GW5/6) x (P48) x L550) x Gaumchii2]	ICRISAT, Hyderabad, India
18	GOKCEE	*Kabuli*	A drought tolerant breeding line FLIP 87-8C	ICARDA, Syria
19	Pusa 256	*Desi*	(JG62x 850-3/27)x (L550 x H 208)	IARI, New Delhi, India
20	IG5856	*Kabuli*	Chickpea Landrace from Maan, Jordan	ICARDA, Syria
21	IG5857	*Kabuli*	Chickpea Landrace	ICARDA, Syria
22	DCP 92-3	*Desi*	Selection from Germplasm	IIPR, Kanpur
23	IG5894	*Kabuli*	Chickpea Landrace from Arbil, Iraq	ICARDA, Syria
24	IG5906	*Kabuli*	Chickpea Landrace from As Sulaymaniyah, Iraq	ICARDA, Syria

Allele scoring and genetic diversity analysis

Scoring of fragments was performed manually to determine the presence or absence of a particular allele, generating a scoring matrix for analysis. If the allele was specific and was found mainly in a single genotype, it was denoted as a unique allele. The scoring matrices were used to compute genetic diversity measurements, including polymorphism information content (PIC), expected and observed heterozygosity, and the Shannon information index. The Cervus v3.0 and POPGENE v1.32 tools were employed for the abovementioned calculations. A neighbor-joining (N-J) tree was created utilizing the dice coefficient and 1000 bootstrapping replicates, accomplished using the DARwin program described by Perrier and Jacquemoud-Collet (2006). To evaluate the genetic composition of the genotypes investigated in this study, the STRUCTURE v2.3.3 software, developed by Pritchard et al. (2000), was utilized.

Results

Phenotyping of chickpea genotypes for salt tolerance

This study assessed the salt sensitivity of 24 chickpea genotypes through morpho-physiological parameters. Elevated salt stress significantly reduced various traits, showing a genotype-by-salinity interaction ($P < 0.05$) (Table 2). Key agronomic, yield-related, and physiological traits declined, including DOM, PH, NPPP, 100 SW, SY, RWC, and MSI. A cumulative stress tolerance index (CSTI) was used to classify genotypes based on salt responsiveness, calculated by summing the individual stress response indices (Fig. 1a). Using percentage reductions across traits, genotypes were grouped into four clusters: clusters 1 and 2 exhibited the highest reductions, while clusters 3 and 4 showed minimal reduction. Salt-sensitive genotypes (ICCV-2, DCP92-3, Pusa 256, Gokcee, SBD 377) had pronounced declines, whereas salt-tolerant genotypes (CSG8962, ICCV 10, JG11, IG5857, ICCV00104, ICCV06101, Pusa 72) demonstrated greater resilience (Fig. 1b).

Genome-wide identification and distribution of genic SSR

Functional annotation and analysis of the identified candidate genes revealed their roles in various biotic and abiotic stress responses. We could identify critical genes that are reported to be involved in salinity, including K^+ transporter-like protein HAK/KUP transporter, E3 ubiquitin-protein ligase, peroxidase, amine oxidase, cysteine-rich knottin fold-containing protein, sucrose nonfermenting-like protein and transcription factors like WRKY, ERF and MYB family TFs. These candidate genes for salinity responsiveness were mined for SSRs, and we could identify 158 cg-SSR motifs within different regions of salt-responsive genes. Details about the genes containing these SSR repeat motifs, including their putative functions, the number and types of

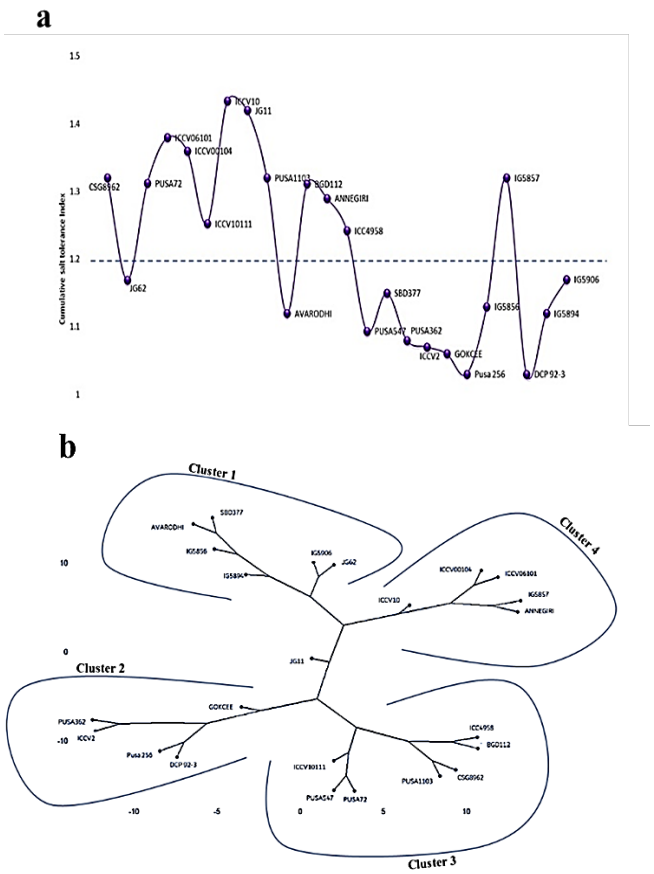

Fig. 1. (a) Classification of chickpea genotypes using CSTI (cumulative salt tolerance index) and (b) Clustering of genotypes based on the morpho-physiological traits

repeat motifs, and their specific locations, are given in Table 3. When considering the distribution of these SSR motifs, we observed that the majority were mononucleotide (32%) and dinucleotide (32%) repeats, followed by trinucleotide (18%), tetranucleotide (11%), pentanucleotide (4%), and hexanucleotide (3%) repeats (Fig 2 a). We identified 44 different types of SSRs, 20 of which appeared only once, while the remaining 24 SSR motifs were observed 2 to 29 times. Among these, mono-nucleotide (A) and (T) motifs were most abundant, followed by dinucleotide motifs (AT) and (TA) (Fig. 2b).

Validation of candidate gene based SSRs (cg-SSR)

Out of 158 cg-SSRs, primers were designed for validation for 20 different salt-responsive cg-SSRs. Among 20 distinguished loci, 17 were amplified, of which 15 were polymorphic. We finally used 15 cg-SSR loci to analyze polymorphism in 24 chickpea genotypes (Table 4). These 15 primers obtained clear and precise polymorphic profiles on a 3 % agarose gel profile; the representative gel images for these primers have been provided in (Fig. 3a, b). After manually scoring all the fragments, the scoring matrix was utilized to calculate genetic diversity metrics, including

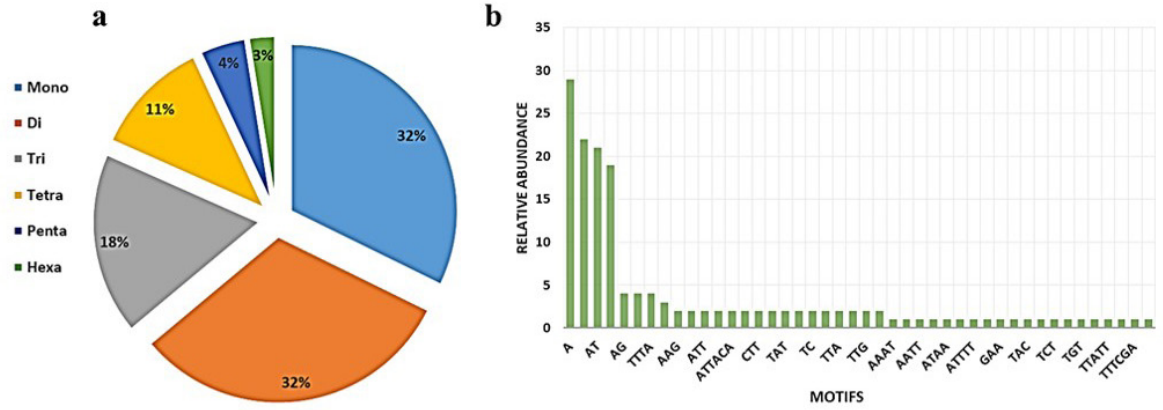

Fig. 2. Distribution of salt responsive cg-SSRs in chickpea, (a) number of different SSR motifs and (b) relative abundance of various SSR motifs

Table 2. Analysis of variance explaining treatment, genotype and treatment by genotype interactions for morpho-physiological traits

Traits	Treatment				Genotype				Treatment: Genotype			
	SS	MSS	F value	DF	SS	MSS	F value	DF	SS	MSS	F value	DF
DOF	423.6736	423.6736	25.96 ns	1	32660.83	1420.036	106.38*	23	1114.493	48.4562	3.63*	23
DOM	14843.36	14843.36	471.11*	1	2558.639	111.2452	29.08*	23	629.3056	27.3611	7.15*	23
PH	28224	28224	2082.1*	1	1280.639	55.68	7.09*	23	1002.667	43.5942	5.55*	23
NPPP	34689.06	34689.06	579.63	1	4452.493	193.5867	6.96	23	4137.438	179.8886	6.46	23
SEED/5POD	189.0625	189.0625	97.58	1	91.6042	3.9828	7.2	23	16.1042	0.7002	1.27ns	23
100SW	258.6736	258.6736	23.46ns	1	4442.326	193.1446	74.49	23	267.4931	11.6301	4.49	23
SY	281784	281784	287.69	1	55562.97	2415.781	12.2	23	46371.64	2016.158	10.19	23
RWC	1308.028	1308.028	58.92	1	2620	113.913	11.36	23	2714.639	118.0278	11.77	23
MSI	175.5625	175.5625	13.34	1	7632.66	331.8548	45.7	23	3564.604	154.9828	21.34	23

(*significant at p <0.05)
DOF = Days of 50% flowering; DOM = Days of maturity; PH = Plant height; NPPP = Number of pods per plant; Number seeds/5 pods; SW = 100 seed weight; SY = Seed yield; RWC = Relative water content; MSI = Membrane stability index (SS: Sum of square; MSS: Mean sum of square; DF = Degree of freedom

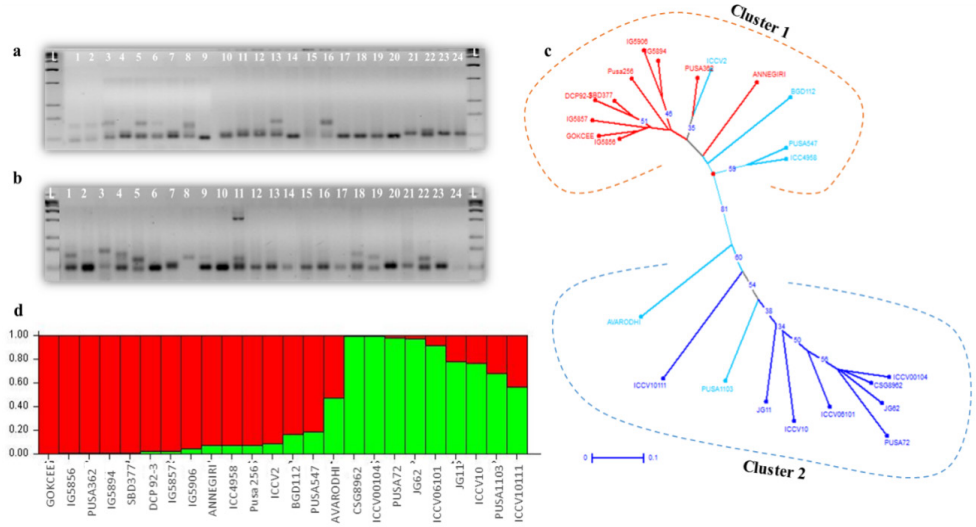

Fig. 3. Representative Cg-SSR banding profile of (a) LOC101501578 and (b) LOC10149980524 markers, where L is 100 bp Ladder, 1-24 represent genotypes, list provided in table 1; (c) radial neighbor-joining tree and (d) genetic structure based on cg-SSR markers

Table 3. Details of salt tolerance gene, respective gene LOC number, motifs with repeat number and location in sequence, primers with Tm and expected band size used for validation

Gene name	Gene Locus Id	(Motif) repeat*	Amplicon Size	Forward (Tm °C)	Reverse (Tm °C)	Function
LOC101501578	Ca3:35034781-35044945	$(TA)_{16}$	199 bp	TCGTTCAATGAGAGATTGTGTGC (59.57)	TCAAAGTTCACGCACATCGC (59.77)	ubiquitin-protein ligase
LOC101489172	Ca3:37183727-37187121	$(TA)_{8}$	183 bp	ACAAGCCTGCTTTTCATCCG (59.12)	TGACACTGACCTACATTTTGTGG (58.87)	WRKY1b transcription factor
LOC101501891	Ca3:38143567-38148493	$(TA)_{13}$	137 bp	TCAGAGCTGATGCACACACC (60.32)	GCGGGGCCATAGATACATCC (60.11)	sucrose nonfermenting-like protein
LOC101510030	Ca3:38334435-38337091	$(AG)_{10}$	169 bp	ACCAATGCCAACTTTCAGAAGG (59.37)	ACTTTTTCAATGAATTCGACCATGC (59.37)	protein-lysine N-methyltransferase EEF2KMT
LOC101502953	Ca3:38789793-38794807	$(TC)_{10}$	176 bp	AGAGAAACGCTTGAAGCATGC (59.8)	AGATAAAATTGGGAGGGGAAAGC (58.71)	E3 ubiquitin protein ligase DRIP2, putative
LOC101493717	Ca3:32977630-32982057	$(TA)_{12}$	143 bp	TCTCGTAGCATCAAACATTCTCC (58.57)	GTATGCCGATTTCCTCCTTCG (58.86)	probable E3 ubiquitin-protein ligase LOG2
LOC101512249	Ca1:33648586-33651426	$(AG)_{8}$	178 bp	AGGTTCCTTATTGTTGGAATCACG (59.3)	AAAACCCCTCAAACCCTCCC (59.81)	UDP-arabinopyranose mutase 3-like
LOC101514640	Ca2:27115033-27120480	$(TG)_{9}$	149 bp	TCTCAGTGCACAAATCTGCC	TAGGTTTGGAGGACGCAACG (60.32)	Peroxidase
LOC101512608	Ca3:23256009-23259476	$(CT)_{8}$	159 bp	CACCGTTCATTCTCTTTCTTTTGC ((59.32)	AGCAATGCAAAACAAAGTGACC (58.8)	Cysteine synthase
LOC101502953	Ca3:38789593-38794877	$(TC)_{10}$	176 bp	AGAGAAACGCTTGAAGCATGC (59.8)	AGATAAAATTGGGAGGGGAAAGC (58.71)	E3 ubiquitin protein ligase DRIP2-like
LOC101499262	Ca4:19831460-19835276	$(AT)_{13}$	172 bp	TTGTGGAGCAAGAGCCTCC (59.62)	TGCTCCTCCTACATTTTATTTTGGG (59.34)	Peroxidase
LOC101502259	Ca6:4811024-4811977	$(TA)_{7}$	137 bp	CCAATGTAGTTTGGTTATGGTGC (58.26)	CTAGAACCAATTTTAACCACACACC (58.56)	uncharacterized protein
LOC101509535	Ca6:23104510-23115495	$(TA)_{23}$	214 bp	GCCTTGATGGTAGATTGTCGC (59.4)	AATCATCGATGACCAACTATTTATGC (58.19)	Glucan endo-1,3-beta-d-glucosidase
LOC101500576	Ca6:53819316-53822661	$(TA)_{8}$	141 bp	AGATTGTAAAATTGTGGTTGTCACC (58.49)	TAGTTTGAAGGTGCAGCAGC (58.76)	cyanogenic beta-glucosidase
LOC101491799	Ca7:5708466-5713083	$(TA)_{14}$	153 bp	TGTGCACGTTTAATTTGTTCAGC (59.21)	CAGCTCGCGCATCAAATCC (60.01)	Amine oxidase
LOC101503983	Ca1:12729834-12733448	$(AAT)_{10}$	160 bp	TTTGATATGAACCAGCGGCG (58.99)	GCCAAAAGGAAAAGCCCTCC (59.68)	protein trichome birefringence-like 39
LOC101499805	Ca6:18298741-18299470	$(TAT)_{9}$	150 bp	CAGCTTCCCCAATCCTATCCC (59.93)	AACACACAGAGGAAGCTCCG (59.97)	ethylene-responsive transcription factor ERF109-like
LOC101491799	Ca7:5708466-5713083	$(TCT)_{8}$	112 bp	TCAGGAGCCCATATCAACGG (59.24)	GCAGAAACAAAATGTCATTCTCGG (59.38)	Amine oxidase
LOC101501430	Ca7:16785641-16792934	$(TAT)_{7}$	105 bp	GATGTGGGTTGGGGAAGAGG (60.03)	GTTTCCCGCGAACCAATTCC (60.11)	isoflavone reductase homolog
LOC101491013	Ca3:37219529-37220770	$(ATTA)_{6}$	191 bp	TTCAGTTCCTAAGCAAGCAGG (58.22)	CACCTAAGCCTACAACTCTTACC (58.24)	myb transcription factor MYB64

Table 4. Diversity statistics for Cg-SSR loci studied in 24 chickpea genotypes

S. No.	Locus	Allele Size	na*	ne*	I*	Ho	He	PIC
1	LOC101501578	130–180	2	1.8	0.6365	0.167	0.454	0.346
2	LOC101489172	120–180	2	1.4922	0.5117	0.417	0.337	0.275
3	LOC101501891	140–200	2	1.9862	0.6897	0.583	0.507	0.373
4	LOC10151003	130–180	2	1.8824	0.6616	0.25	0.479	0.359
5	LOC101502953	170–200	2	1.9459	0.6792	0	0.496	0.368
6	LOC101514640	90–160	2	1.8824	0.6616	0	0.479	0.359
7	LOC101512608	120–180	2	2	0.6931	0	0.511	0.375
8	LOC101502953	180–240	2	1.9836	0.689	0	0.507	0.373
9	LOC101500576	160–220	2	2	0.6931	0	0.511	0.375
10	LOC101491799	160–200	2	1.9665	0.6846	0.087	0.502	0.371
11	LOC101503983	160–220	2	1.8824	0.6616	0.167	0.479	0.359
12	LOC101499805	150–200	2	1.3318	0.4154	0.292	0.254	0.218
13	LOC101491799	80–100	2	1.8	0.6365	0	0.454	0.346
14	LOC101501430	100–150	2	1.9459	0.6792	0	0.496	0.368
15	LOC101491013	100–200	2	1.682	0.5954	0.304	0.414	0.323
	Mean		2	1.838753	0.639213	0.151133	0.458667	0.345867

na = Observed number of alleles; ne = Effective number of alleles; I = Shannon's information index; Ho = Observed heterozygosity; He = Expected heterozygosity; PIC = Polymorphic information content

polymorphism information content (PIC), expected and observed heterozygosity, and Shannon's information index. These calculations were performed using Cervus v3.0 (Kalinowski et al. 2007) and POPGENE v1.32 (Yeh and Boyle, 1997). The PIC values for the primers ranged from 0.21 to 0.37, with an average of 0.34, while heterozygosity (He) lies between 0.25 and 0.51, with an average of 0.45. Shannon's Information Index (I) varied from 0.51 to 0.69, with an average value of 0.63 (Table 4).

Estimation of genetic diversity using cg-SSR

Dissimilarity analysis was conducted to assess genetic diversity using data from 15 polymorphic cg-SSRs profiles for 24 genotypes. A DARwin program (Perrier and Jacquemoud-Collet, 2006) was employed to construct a neighbor-joining (N-J) tree using the Dice coefficient and bootstrapping with 1000 replicates. Further, explore the genetic structure of the genotypes by using STRUCTURE v2.3.3. Based on the fragments generated, the neighbor-joining (N-J) tree categorized the genotypes into two clusters, illustrating the extent of variation among the selected accessions in this study. Cluster-I consists of 14 genotypes, including four that were moderately tolerant and ten that were sensitive. Meanwhile, Cluster-II comprised ten genotypes, with two moderately tolerant and eight tolerant (Fig 3 c). The Bayesian genetic structure and admixture analysis of the accessions revealed the presence of two distinct gene pools, represented by red and green colors, among the genotypes used in the current study (Fig 3 d). So, it is evident that the developed cg-SSRs from salt-responsive genes can differentiate the genotypes because of their salinity tolerance.

Discussion

With salt stress posing a growing threat to global agriculture and food security, it is imperative to develop salinity-resilient chickpea cultivars (Tarolli et al. 2024). In this step, we identified and developed salt-responsive candidate gene-derived simple sequence repeat (SSR) markers (cg-SSRs). These markers hold promise for enhancing salt stress tolerance. Chickpea, a key grain legume, is highly sensitive to salinity, which disrupts osmotic balance by causing salt accumulation in vacuoles of xylem meristems. To cope, plants maintain osmotic balance, regulate ion concentrations, detoxify reactive oxygen species (ROS), and activate genes related to cellular adaptation, cell wall maintenance, and preventing water loss (Zhou et al. 2024). Salinity negatively impacts antioxidant activity, chlorophyll fluorescence, lipid peroxidation, biomass, and seed yield. Gene regulatory networks and genomic regions associated with salinity stress in chickpea have been explored using bi-parental mapping, uncovering genetic loci influenced by background variations (Soren et al. 2020; Atieno et al. 2021). The complex interplay of genes in stress responses makes it challenging to pinpoint a single gene responsible for salinity tolerance. Many genes and gene families respond to salinity

by modulating physiological or morphological traits. These include HKT, Na^+/H^+ antiporter, $Na^+/Ca2^+$ exchanger, serine/threonine protein kinase, peroxidase, calcium-dependent and dehydration response genes DHN3 and NAC7(Awaly et al. 2025). Regulatory genes like those in the DREB family enhance stress tolerance by activating stress-inducible genes, often ABA-independently, via specific cis-elements in promoter regions (Soren et al. 2020; Kumar et al. 2021). To effectively incorporate candidate gene data into breeding salt-tolerant chickpeas, a rapid integration mechanism is essential. Simple sequence repeats (SSRs), are a widely available, cost-effective and accessible genotyping tool. As a crucial molecular tool in genetics and biological research, candidate gene-based SSRs provide enhanced efficiency and specificity, making them valuable targets for future breeding programs.

By utilizing diverse salt-responsive genes across functional categories, we successfully generated markers located in linkage groups 3 and 6, with the potential to transform genomics-assisted breeding (GAB). These findings offer valuable insights for advancing research on plant stress tolerance. Identified genes encode various proteins, including kinases (MAPKs, CDPKs, SnRK1) and transcription factors (WRKY, ERF, MYB), all of which are pivotal in the salinity stress response. WRKY transcription factors are key regulators in plant stress adaptation (Sun et al. 2020; Erpen et al. 2018), while MYB plays a crucial role in plant growth and abiotic stress tolerance, including salinity (Dossa et al. 2020). These findings reinforce their significance in enhancing stress resilience in plants. Similarly, SnRKs play a crucial role in regulating and transmitting stress signals across cellular compartments in response to abiotic stress (Feng et al. 2022). E3 ubiquitin ligase genes contribute to hormonal signaling and environmental stress adaptation, proven to enhance salt tolerance in rice (Li et al. 2021). Similarly, peroxidases play a vital role in antioxidant defense and salt stress response in plants. The current study identified salt stress-responsive candidate genes by mining quantitative trait locus (QTL) regions and differentially expressed genes (DEGs). The development and validation of candidate gene-based SSR markers (cg-SSRs) offer a valuable genomic resource for distinguishing salt-tolerant and sensitive chickpea genotypes. Microsatellite markers, such as simple sequence repeats (SSRs), play a crucial role in molecular profiling and genetic diversity analysis (Misganaw and Abera 2017) due to their high polymorphism, co-dominance, and even genomic distribution. Scanning candidate gene sequences for SSR loci revealed mono- (32%) and dinucleotide (32%) repeats as most abundant, followed by tri- (18%), tetra- (11%), penta- (4%), and hexa-nucleotide (3%) repeats. Mono-nucleotide (A, T) motifs were predominant, followed by dinucleotide (AT, TA) motifs. While replication slippage does not occur in trinucleotide repeats, it is common in mono- and dinucleotide repeats, with tri-nucleotide repeats exhibiting greater genomic diversity and complexity.

Our study validated salt-responsive cg-SSRs to distinguish chickpea genotypes based on salt tolerance. The hypothesis linked germplasm variation to length polymorphisms at cg-SSR loci. Of the 20 cg-SSRs analyzed, 15 produced polymorphic bands, while three failed to amplify and two were monomorphic, confirming diversity in salt-responsive gene expression. The mean PIC value (0.34) exceeded previous reports in wheat and rice (Molla et al. 2015; Singh et al. 2018). A UPGMA dendrogram revealed two clusters: one comprising salt-tolerant genotypes and the other salt-sensitive ones, aligning with prior findings in wheat and rice (Molla et al. 2015; Singh et al. 2018). Genotypes such as ICCV 10, JG11, and Pusa 72 demonstrated strong salt tolerance, consistent with earlier reports (Kumar et al. 2017; Kumar et al. 2020). These findings underscore the role of salt-responsive microsatellite loci in shaping chickpea genotypic differences. The cg-SSRs identified here offer valuable genomic resources for breeding salt-tolerant chickpeas and identifying salt-tolerance loci in other crops. Further validation at the microsatellite locus level is needed to refine these insights.

Authors' contribution

Conceptualization of research (CB); Designing of the experiments (NK, BSP, CB); Contribution of experimental materials (CB); Execution of field/lab experiments and data collection (NK, SS, MR); Analysis of data and interpretation (RKV, KHMS, SS); Preparation of the manuscript (NK, CB).

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