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Trans-QTL Alliance of HKT1 and PHL7 Modulate Salinity Stress Tolerance and Enhance Crop Yield Endurance

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ABSTRACT

Salinity stress can cause significant yield losses in crops because of its major impact on reproductive success. The complexity of salinity stress responses, particularly their tissue- and cell-specific regulation, continues to challenge the translation of molecular insights into tangible crop yield improvements. In the present study, the authors deployed a genomic strategy combining a genome-wide association study, regional association analysis, QTL mapping, fine mapping, and map-based cloning to delineate a pair of novel *CaPHL7* and *CaHKT1* alleles that regulate yield under salinity stress. The selected contrasting accessions, developed near-isogenic lines (NILs), overexpressed chickpea lines and complemented *Arabidopsis* lines collectively underscore the functional significance of the identified alleles in relaying yield endurance under salinity stress conditions. Functional characterisation of the genes revealed the intricate transcriptional regulation of *CaHKT1* by *CaPHL7*, which influences the degree of salinity stress tolerance. Furthermore, in our efforts to enhance yield endurance, we discovered a novel regulatory role for the phosphorus (P) starvation-responsive gene (*PHL7*) in legumes, facilitating salinity stress adaptation. This study provides the first functional validation of a trans-QTL regulatory model in chickpea, where *CaPHL7*, located on one chromosome, transcriptionally activates *CaHKT1* on a separate chromosome. The regulatory mechanism plays a key role in excluding sodium from the transpiration stream, thereby protecting reproductive processes from salinity-induced damage and mitigating yield penalties. This inter-locus regulation explains yield stability and offers useful insights that may be considered in future efforts to enhance salt resilience in chickpea.

1 | Introduction

Chickpea (*Cicer arietinum*) is vital in global agriculture and dietary regimes because of its cosmopolitan growth habit, economic importance, affordable nutritional value, and soil-enriching ability (Herridge et al. 1995; Mohanty et al. 2022; Mehrotra et al. 2023). As a leguminous crop, chickpea engages in symbiotic nitrogen fixation, thereby improving soil fertility and contributing to sustainable agricultural systems (Herridge et al. 1995). Its culinary versatility makes it

a dietary staple, particularly in semi-arid tropical regions (Mehrotra et al. 2023). However, increasing soil salinization due to seawater intrusion, excessive irrigation, and climate change has affected chickpea productivity considerably (Atieno et al. 2017; Munns and Tester 2008; Jiang et al. 2018; Cao et al. 2019). Currently, over 20 million hectares of farmland globally are affected by high salinity, and the area is expanding by 10% annually, with 4.4% of topsoil and 8.7% of subsoil across 118 countries classified as saline (FAO 2021; Qadir et al. 2014; Shrivastava and Kumar 2014).

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Chickpeas are more sensitive to salinity stress than mainstream cereals. However, they exhibit considerable phenotypic variability, with sensitive genotypes faltering at 25 mM NaCl, whereas tolerant genotypes tolerate salinity stress as high as 100 mM (Vadez et al. 2007; Flowers et al. 2010; Turner et al. 2013). Salinity affects chickpea growth, yield, and quality adversely, causing an estimated 8%–10% production loss (Flowers et al. 2010). It affects all stages of chickpea development, including germination (Khalid et al. 2001), seedling growth (Pujol et al. 2000), vegetative development (Khan et al. 2015), and reproduction (Vadez et al. 2007, 2012; Samineni et al. 2011; Turner et al. 2013; Khan et al. 2017). Salinity stress disrupts plant physiology through both osmotic stress and ion toxicity. The osmotic stress caused by reduced soil water potential which ultimately hampers water uptake by the root and causes drought-like symptoms. On the other hand, ion toxicity, especially due to Na⁺ accumulation, can induce tissue damage by programmed cell death and autophagy (Munns and Tester 2008; Van Zelm et al. 2020; Demidchik et al. 2010; Katsuhara 1997). To mitigate these effects, plants employ multiple mechanisms, including Na⁺ exclusion from the shoot via specialised transporters like the HKT family. Based on transport characteristics, HKTs are classified into two groups: class I, which transports Na⁺ selectively due to a serine (Ser) residue in the first pore domain, and class II, which allows transport of both Na⁺ and K⁺, due to a glycine (Gly) residue (Mäser, Eckelman, et al. 2002). In several crops, HKT1-type symporters have been reported to play significant roles in shoot Na⁺ exclusion. For example, *Arabidopsis athkt1* mutants accumulate excess Na⁺ in shoots. Multiple QTLs harbouring *HKT1* have been characterised in several crops, such as *SKC1/SalTol* in rice (Ren et al. 2005), *Kna1*, *Nax1*, and *Nax2* in wheat (Huang et al. 2006; Munns et al. 2012), *ZmNC1* in maize (Zhang et al. 2018), and *SIHAK20* in tomato (Wang, Hong, et al. 2020). HKT1 has been a focal point for salinity research and crop breeding for several decades, yet it has not led to any major breakthroughs so far. Although introgression of *Nax2* in wheat showed a 25% yield increase under salinity in one environment (Munns et al. 2012), the broader success and reproducibility of such interventions have been variable and context-dependent. Similarly, *ZmNC3* in maize was shown to reduce shoot Na⁺ by up to 80% (Zhang et al. 2023), though further validation across diverse environments is necessary.

In chickpeas, genetic dissection of salinity tolerance remains limited, with most studies focusing on low-resolution QTLs and vegetative trait screening under controlled environments (Serraj et al. 2004). Additionally, studies have shown that chickpea lines with comparable biomass can display markedly different seed yields under salinity stress, suggesting that vegetative tolerance does not always translate into yield stability (Vadez et al. 2006, 2007). Furthermore, constitutive overexpression of *HKT1* in crops has often been unsuccessful due to the need for cell-specific expression, underscoring the importance of investigating the regulatory mechanisms that control *HKT1* expression (Møller et al. 2009; Plett et al. 2010; Ali et al. 2012).

To address the research gaps above, the present study investigated salinity tolerance from a yield perspective and aimed to identify upstream genetic factors and superior gene(s)/allele(s) that could minimise yield loss under salt stress. A comprehensive

genomic approach combining genome-wide association study (GWAS), regional association, QTL/fine mapping, and map-based cloning studies identified two novel alleles, *CaHKT1* and *CaPHL7*. Functional validation using NILs, overexpression lines, and complemented *Arabidopsis* mutant confirmed their role in salt tolerance. Notably, we report, for the first time, that *CaPHL7* transactivates *CaHKT1* under saline conditions, enabling Na⁺ exclusion and protecting chickpea reproduction. The molecular tags have high translational value in the breeding of salt-resilient, high-yielding chickpea varieties.

2 | Results

2.1 | GWAS and Regional Association Analysis Scan Potential Genomic Loci Associated With Yield Under Salinity Stress in Chickpea

The salinity stress response of 291 chickpea accessions was evaluated using the Salinity Yield Index (SYI), which showed a wide range of trait values (SYI: 0.22–0.95), with a coefficient of variation (CV) of 23.2%, demonstrating a normal phenotypic distribution and a broad-sense heritability of 83% (Figure S1 and Tables S1–S3). High-coverage Genotyping by Sequencing (GBS) of 291 chickpea accessions identified approximately 5.7 million genome-wide single-nucleotide polymorphisms (SNPs), which were filtered to 83,176 high-quality SNPs mapped across eight chromosomes for subsequent genetic relatedness and GWAS analyses (Figure 1A; Table S4). Phylogenetic analysis based on an unrooted neighbour-joining (NJ) tree clustered the 291 chickpea accessions into three major groups based on their overall biological status (Figure 1B). The genome-wide pattern of the population structure, determined using ADMIXTURE, suggested that three populations ($K = 3$) serve as the optimal cluster for the diversity panel (Figure 1C). The outcomes are supported further by Principal Components Analysis (PCA) findings, which inferred an almost similar clustering pattern among the 291 chickpea accessions (Figure 1D). Hence, population stratification was corrected by incorporating the first three principal components as covariates in the GWAS association models. GWAS was performed by integrating the genotyping information of 83,176 high-quality SNPs with the multi-environment replicated phenotypes of SYI and the genetic relatedness information (PCA, Population structure, and kinship matrix) of 291 accessions. This analysis identified three genomic loci, *Ca1_6750456* (A/G), *Ca1_6744541* (C/T) and *Ca8_16300227* (G/A) mapped on chromosomes 1 and 8, exhibiting a significant association with SYI ($< 10^{-9} P$ and 30.2%–34.5% R^2) in chickpeas (Figure 1E). SYI-associated SNP loci identified on chromosomes 1 and 8 were annotated in the upstream regulatory regions (URRs) of *CaPHL7* and *CaHKT1* in chickpeas. The GWAS findings were validated further through gene-by-gene regional association analysis, identifying the 10- and 100-kb genomic regions on chromosomes 1 (6.74–6.75 Mb, mean $R^2 = 0.91$) and 8 (16.30–16.40 Mb, mean $R^2 = 0.95$), respectively. The genomic intervals flanking either side of GWAS-derived SNPs in *CaPHL7* and *CaHKT1* showed significant linkage disequilibrium (LD) resolution (Figure 1F,G). Within the LD blocks, regional association analysis also demonstrated a strong association of the three GWAS-derived SNPs of *CaPHL7* and *CaHKT1* with SYI in chickpea.

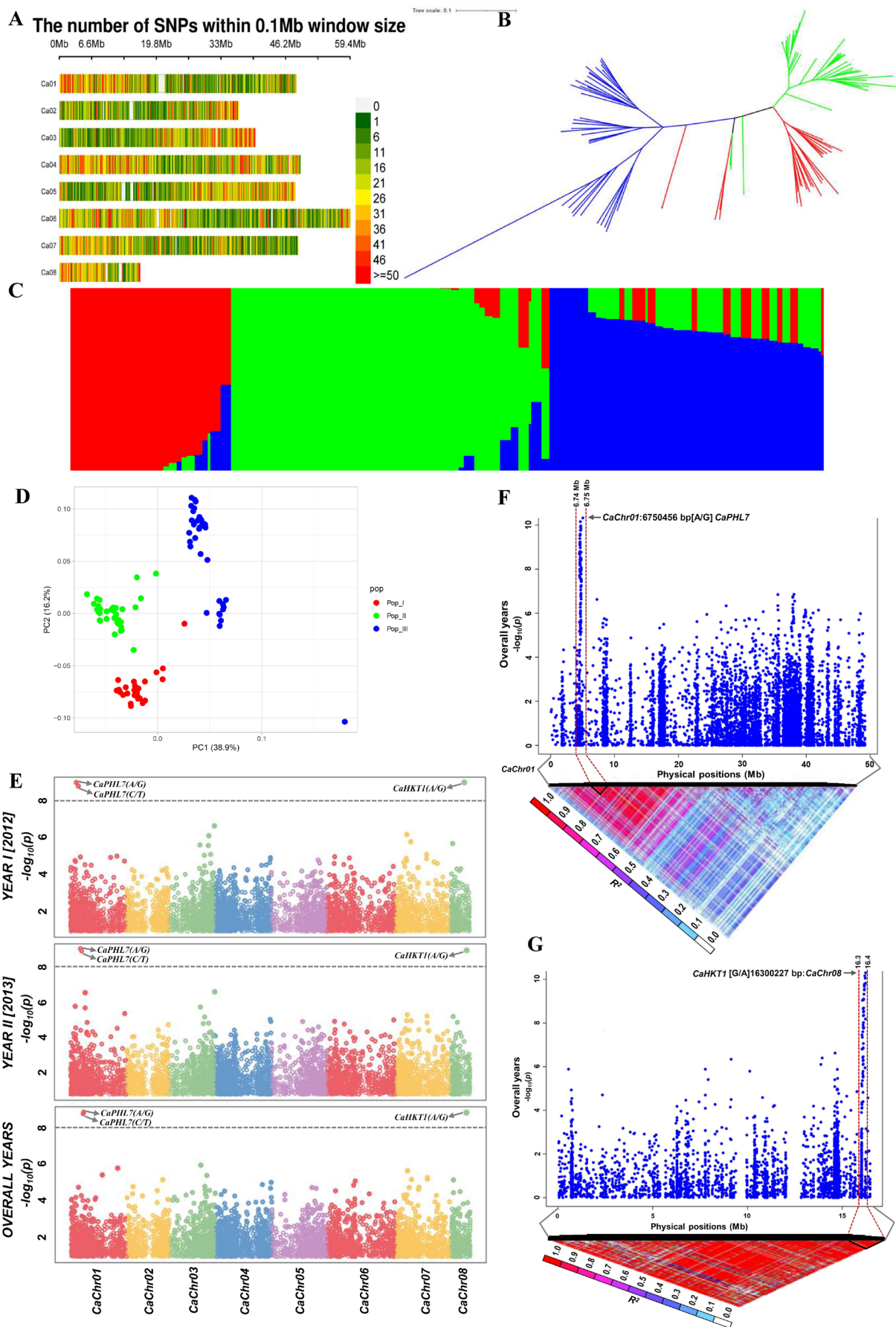


FIGURE 1 | Legend on next page.

FIGURE 1 | High-throughput genotyping, population stratification, GWAS and regional association analysis scan potential genomic loci associated with yield stability during salinity stress in chickpea. (A) Genome-wide SNPs from 291 salinity stress-responsive chickpea accessions are mapped across eight chromosomes (Varshney et al. 2013), with a colour gradient indicating SNP density per 0.1 Mb window. (B) Unrooted phylogenetic tree representing genetic relationships and molecular diversity among 291 chickpea accessions belonging to a diversity panel. (C) Population genetic structure analysis grouped 291 chickpea accessions into three major populations, represented by distinct colours, with each vertical line denoting an individual accession. (D) Principal component analysis (PCA) differentiates the 291 accessions of a diversity panel into 3 distinct population groups (POP I, POP II, POP III). (E) Manhattan plots show significant marker-trait associations (MTAs) for the salinity yield index (SYI). Each dot represents genome-wide SNPs across eight chickpea chromosomes (X-axis), with their height indicating $-\log_{10}(P)$ values (Y-axis). The skyscraper SNPs above the dotted threshold line highlight significant MTAs for SYI. (F, G) Regional association analysis and high-resolution linkage disequilibrium (LD) heat maps highlight 10 and 100 kb genomic regions on chromosomes 1 (Ca1) and 8 (Ca8) surrounding SYI-associated SNPs.

2.2 | High-Resolution Molecular Mapping Reveals Two Key QTL Genomic Regions Governing Yield Under Salinity Stress in Chickpea

To validate the GWAS results, molecular mapping of major salinity-responsive QTLs was conducted to identify the causal genes underlying salinity tolerance in chickpeas. For high-resolution QTL mapping, a recombinant inbred line (RIL) mapping population comprising 216 individuals was generated by inter-crossing two chickpea genotypes with contrasting SYI: ICC 9942 (high SYI, Salt Tolerant/ST) and ICC 6306 (low SYI, Salt Sensitive/SS) (Figure 2A). Multi-environment replicated field phenotyping of 216 RILs revealed a broad range of SYI values from 0.36 to 0.97, with a CV of 19.6% demonstrating a normal phenotypic distribution, and a broad-sense heritability of 86% (Figure S1 and Table S3). An ultra-high-density genetic linkage map of 82,247 SNPs mapped across eight chickpea chromosomes with an average density of 0.013 cM (4.22 kb) was constructed. By integrating the mapped genotyping data with the SYI trait phenotypes in the 216 RILs, two major QTLs were identified on chromosomes 1 (*CaqSYI1.1*) and 8 (*CaqSYI8.1*) (Figure 2B and Table S5). The QTLs were validated across two environments and explained 35.8%–37.6% phenotypic variation, with a logarithm of the odds (LOD) score of 14.7–21.3.

2.3 | Fine Mapping and Map-Based Cloning Delineate Two Causal Genes *CaPHL7* and *CaHKT1* of Major QTLs Controlling Yield Under Salinity Stress in Chickpea

Two key QTL genomic regions, *CaqSYI1.1* (10.46 kb) and *CaqSYI8.1* (6.70 kb), were targeted for fine mapping and map-based cloning to narrow down the QTLs to the causal gene(s) responsible for salinity stress tolerance in chickpea. Salinity-sensitive (*SS-NIL^{CaqSYI1.1}/SS-NIL^{CaqSYI8.1}*) and salinity-tolerant (*ST-NIL^{CaqSYI1.1}/ST-NIL^{CaqSYI8.1}*) BC₄F₅ NILs were generated, exhibiting approximately 88%–90% recovery of the recurrent parental genomes. To fine-map *CaqSYI1.1*, the SNP genotyping information of the 10.46 kb QTL interval was integrated with the phenotyping data of 311 individuals of an F₂ mapping population (*ST-NIL^{CaqSYI1.1} × SS-NIL^{CaqSYI1.1}*). This identified 12 recombinants for the *CaqSYI1.1* QTL, which narrowed down to a 5.55 kb genomic region on chromosome 1 (Figure 2C). Structural and functional annotation of the region revealed a single MYB transcription factor gene, *CaPHL7* (*Ca_07939*), which is strongly associated with the SYI trait in chickpea. The upstream regulatory region (URR)-SNP [*Ca1_6750456/CaSNP3632* (A/G)]

co-inherited with *CaPHL7* showed a lack of recombination in six promising NIL recombinants (Figure 2D). This confirmed the pivotal role of *CaPHL7* in salinity stress tolerance in chickpea.

To fine-map *CaqSYI8.1*, the SNP genotyping information within a 6.7 kb QTL region was integrated with the phenotyping data from 331 mapping individuals of an F₂ population (*ST-NIL^{CaqSYI8.1} × SS-NIL^{CaqSYI8.1}*). This identified 10 recombinants for *CaqSYI8.1* QTL, which narrowed down to a 6.16-kb genomic region on chromosome 8 (Figure 2E). Furthermore, high coverage (~120×) multiplex amplicon resequencing of 6.16 kb *CaqSYI8.1* QTL in the parental genotypes, as well as in each of the homozygous ST-NIL and SS-NIL, identified 28 SNPs. QTL region-specific association analysis of 291 accessions and progeny testing of 10 recombinants narrowed the 6.16 kb *CaqSYI8.1* QTL into a 3.55-kb region in five promising recombinants of NILs (Figure 2F). This genomic region harbours the GWAS-derived *CaHKT1* gene (*Ca_15550*), which is strongly associated with salinity stress tolerance (SYI) in chickpea. A URR-SNP [*Ca8_16300227/CaSNP82237* (G/A)] co-inherited with *CaHKT1* showed a lack of recombination in the five promising NIL recombinants, ascertaining the critical role of *CaHKT1* in salinity tolerance. In summary, this combinatorial genomic approach, integrating GWAS, regional association studies, high-resolution QTL mapping, and map-based cloning, identified two key genes, *CaHKT1* and *CaPHL7*, as major regulators of salinity stress tolerance in chickpea.

2.4 | Natural Variation in Shoot Na⁺ Content Determines Chickpea Salt Tolerance

To investigate salinity tolerance in chickpea, contrasting salinity-tolerant lines (*ST-NIL^{HKT1}*, ICC 9942) and salinity-sensitive lines (*SS-NIL^{HKT1}*, ICC 6306) were grown under control and saline conditions. Tolerant lines (*ST-NIL^{HKT1}* and ICC 9942) exhibited reduced vegetative damage and limited yield loss under salinity stress, whereas the sensitive lines showed pronounced leaf scorching and withering starting from older leaves (Figure 3A). Maintaining low shoot Na⁺ content and an ideal Na⁺/K⁺ ratio is linked to diverse salt tolerance in various crops (Ren et al. 2005; Byrt et al. 2007; Henderson et al. 2018; Zhang et al. 2018, 2023; Cao et al. 2019). In the contrasting NILs, ionic content measurements via Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) revealed no significant differences under control conditions. However, under salinity, *ST-NIL^{HKT1}* maintained significantly ($p \leq 0.001$) lower shoot Na⁺, unchanged K⁺ level, and a lower Na⁺/K⁺ ratio than

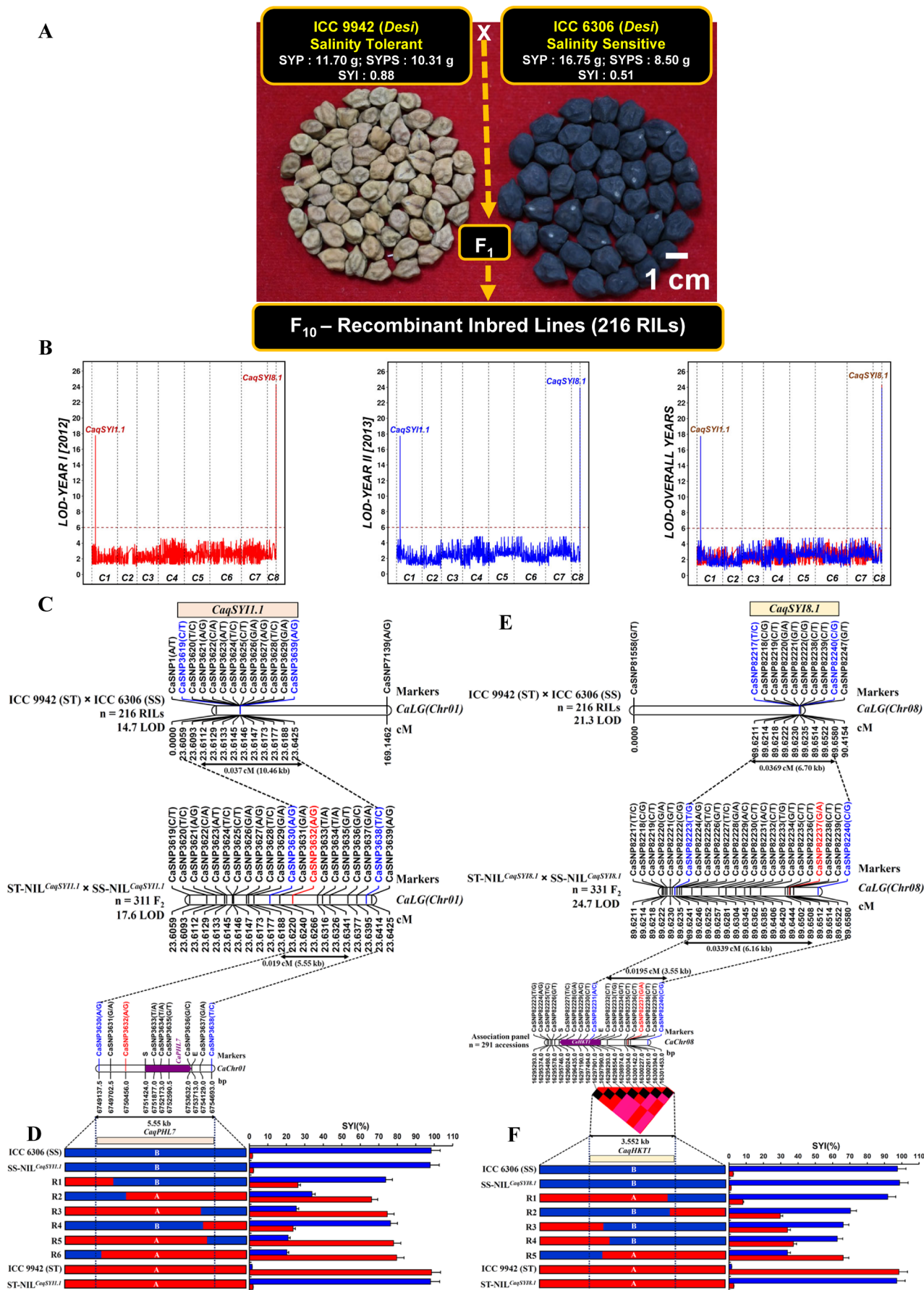


FIGURE 2 | High-resolution QTL mapping, fine-mapping, and map-based cloning delineated two key genomic regions, CaPHL7 and CaHKT1, as major determinants of yield stability under salinity stress in chickpea. (A) Generation of a chickpea recombinant inbred line (RIL) mapping population for salinity stress response, (B) High-resolution QTL mapping presents LOD score distribution plots for reaction norms, arranged by SNP allele-specific order over multiple years. Dotted lines mark the LOD score threshold from a 1000-permutation test. Red and blue plots above the threshold indicate QTL regions linked to the salinity yield index (SYI) for 2012 and 2013, respectively, with an overlaid plot highlighting the consensus locus from multi-environment QTL analysis. (C) Fine-mapping of the *CaqSY11.1* QTL genomic region using the F_2 mapping population ($ST-NIL^{CaqSY11.1} \times SS-NIL^{CaqSY11.1}$) of 311 individuals demonstrating a wider SYI trait variation. The said fine-mapping followed by the integration of high-density genetic linkage map with the corresponding physical map, scale-down the 10.46 kb (0.0366 cM) QTL interval to 5.55 kb (0.0194 cM) genomic region harbouring a single *CaPHL7* gene. The markers flanking the *CaqSY11.1* and tightly linked to *CaPHL7* were demarcated with blue and red colour, respectively. (D) The progeny testing by integrating the multi-environmental replicated phenotyping of the homozygous recombinant and non-recombinant progenies derived from the mapping population ($ST-NIL^{CaqSY11.1} \times SS-NIL^{CaqSY11.1}$) along with their mapping parental lines and contrasting NILs for salinity stress response. At this QTL interval, CaSNP3632(A/G) is tightly linked to *CaPHL7* and demonstrates zero recombination with the target loci in the six recombinant NILs. The genomic constitution of ST-NIL and SS-NIL were represented with 'A' and 'B' respectively, whereas the horizontal lines indicate the average phenotypic constitution for the SYI ($n = 5$ to 8). (E) Fine-mapping of the *CaqSY18.1* QTL using the F_2 mapping population ($ST-NIL^{CaqSY18.1} \times SS-NIL^{CaqSY18.1}$) of 331 individuals exhibiting a wider SYI trait variation. The said fine-mapping followed by the integration of high-density genetic linkage map with the corresponding physical map, scale-down the 6.7 kb (0.0369 cM) QTL interval to 6.16 kb (0.0339 cM) genomic region. Subsequently, a regional association analysis with 291 diverse chickpea accessions delineated a 3.55 kb (0.0195 cM) genomic region harbouring a single *CaHKT1* gene. The SNPs flanking the *CaqSY18.1* QTL and tightly linked to *CaHKT1* gene are demarcated with blue and red colour, respectively. (F) The progeny testing by integrating the multi-environmental replicated phenotyping of the homozygous recombinant and non-recombinant progenies derived from mapping population ($ST-NIL^{CaqSY18.1} \times SS-NIL^{CaqSY18.1}$) along with their mapping parental lines and contrasting NILs for salinity stress response. At this QTL interval, CaSNP82237(G/A) is tightly linked to *CaHKT1* and demonstrates zero recombination with the target loci in the five recombinant NILs. The genomic constitution of ST-NIL and SS-NIL was represented with 'A' and 'B' respectively, whereas the horizontal lines indicate the average phenotypic constitution for the SYI ($n = 5$ to 8).

SS-NIL^{HKT1} (Figure 3C–E). In both the NILs, root Na^+ and Na^+/K^+ ratios showed patterns opposite to that observed in the shoots. That means root Na^+ accumulation of *ST-NIL^{HKT1}* is significantly higher ($p \leq 0.001$) than that of *SS-NIL^{HKT1}*, which may have resulted in the less Na^+ accumulation in the shoot of *ST-NIL^{HKT1}*. This result indicates that the root to shoot Na^+ transport rate is significantly higher in *SS-NIL^{HKT1}* compared to in *ST-NIL^{HKT1}*. Such a differential transport rate determines the variation in shoot Na^+ , which subsequently defines the different degree of salinity stress tolerance in chickpea.

2.5 | *CaHKT1* Encodes for a Na^+ -Preferential Ion Transporter Located in the Plasma Membrane

CaHKT1 functions as a sodium (Na) preferential transporter in crops such as *Arabidopsis*, rice, and wheat (Mäser, Eckelman, et al. 2002; Berthomieu et al. 2003; Rus et al. 2004; Davenport et al. 2007; Horie et al. 2007; Byrt et al. 2007). *CaHKT1* encodes a 533 amino acid-long transmembrane protein with a TrkH domain, and it is clustered phylogenetically with class I HKTs of different crops (Figure S2A,B). Gene family analysis and domain annotation indicated that *CaHKT1* is a class I subfamily of HKT, featuring Ser-84 in the first pore domain, which is a characteristic feature of Na^+ uniporters, in contrast to Gly-bearing Class II HKTs, which act as Na^+/K^+ symporters (Figure S2C) (Mäser, Hosoo, et al. 2002).

In transiently transformed *N. benthamiana* leaf cells, the fluorescence produced by YFP-tagged *CaHKT1* outlined the cell contour with a continuous signal, characteristic of the plasma membrane (Figure 3D). To further evaluate the ion (Na^+/K^+) selectivity and transport activities of *CaHKT1*, the 2-electrode voltage clamp method was performed by injecting a Na^+ /potassium (K^+) gluconate salt into *Xenopus laevis* oocytes expressing

CaHKT1. The *CaHKT1*-expressing oocytes injected with gluconate salt solutions of different Na^+ and K^+ concentrations showed substantial variations in inward and outward currents compared to water (as a control). With an increase in Na^+ gluconate salt concentration from 1 to 30 mM, a significant shift in the reverse potential was observed from -86 to -20 mV in the *CaHKT1*-expressing oocyte cells (Figure 3G). In contrast, no significant shift in the reverse potential was observed with increasing K^+ gluconate salt concentration (1–30 mM) in *CaHKT1*-expressing oocytes (Figure 3H). Overall, the outcomes based on the *Xenopus* oocyte assay confirm that *CaHKT1* is an Na^+ -preferential ion transporter.

2.6 | *CaHKT1* Facilitates Shoot Na^+ Exclusion by Retrieving Na^+ From the Xylem Sap, Thereby Influencing Salt Tolerance Level in Chickpea

Under salinity stress, *CaHKT1* was expressed predominantly in root tissues compared to in shoots and leaves (Figure 3B). The transcript levels were significantly higher in the roots of *ST-NIL^{HKT1}* (~5.8-fold) than in the roots of *SS-NIL^{HKT1}* (~3.8-fold) (Figure 3B). The elevated expression in tolerant lines during salinity stress prompted functional validation of *CaHKT1*. Earlier studies suggest that constitutive overexpression of *HKT1* can lead to deleterious phenotypes due to Na^+ toxicity (Møller et al. 2009). Therefore, root-specific overexpression of *CaHKT1* was performed in the salinity-sensitive chickpea accession (ICC 6306) using *A. rhizogenes*-mediated hairy root transformation (Figure 4A).

The *CaHKT1*-over-expression (OE) lines displayed an approximately 38.45-fold increase in transcript levels, confirming successful transformation (Figure 4B). Under salinity stress, the lines exhibited tolerant phenotypes (Figure 4C). Shoots of

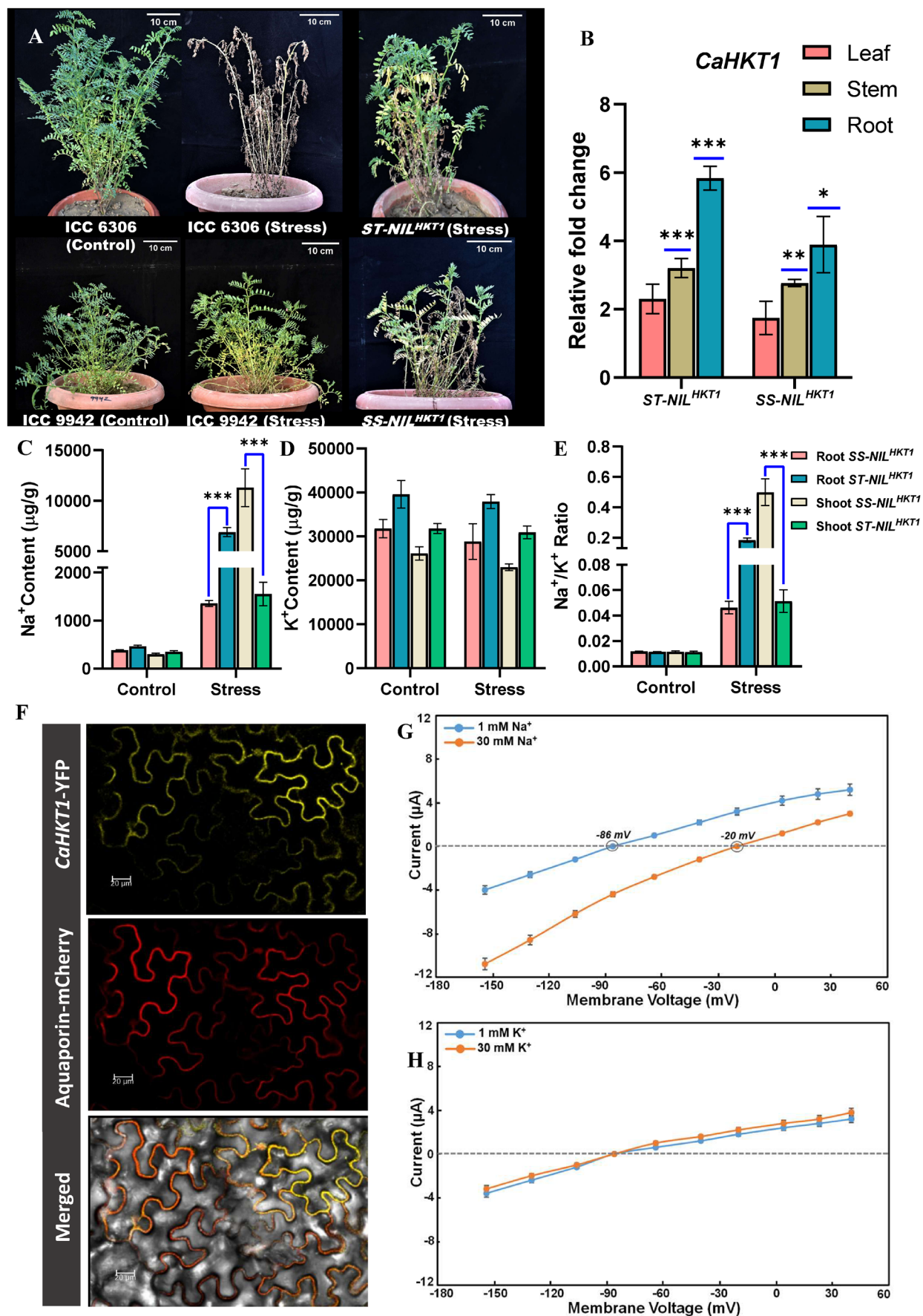


FIGURE 3 | Legend on next page.

FIGURE 3 | Degree of salinity tolerance in the contrasting RIL parental accessions (ICC 6306 and ICC 9942) and NILs varies due to the efficient root Na^+ exclusion by class I Na^+ -preferential HKT family ion transporter *CaHKT1*. (A) Salinity stress-responsive phenotypes observed in the contrasting RIL parental accessions (ICC 6306 and ICC 9942) along with the salinity-sensitive NIL (*SS-NIL^{HKT1}*) and salinity-tolerant NIL (*ST-NIL^{HKT1}*), respectively. The scorched leaves in the plant depicts the intensity of sensitivity of that particular accession/NIL. (B) Represents the fold expression changes of the *CaHKT1* gene under the salinity stress (ST) condition with respect to their controlled counterpart in leaf, stem and root of salinity-sensitive and -tolerant NILs. The values are expressed as the mean $\pm$ S.E., with three biological and technical replicates per sample in the RT-PCR assay. Statistical significance is indicated by Student's *t*-test, where *** $p \leq 0.001$ ** $p \leq 0.01$ and * $p \leq 0.05$. S.E. (Standard Error). (C–E) Relative accumulation of Na^+ content (C), K^+ content (D), and Na^+/K^+ ratio (E), in the root and shoot tissues of contrasting NILs (*SS-NIL^{HKT1}* and *ST-NIL^{HKT1}*) under control and salinity stress condition. Data represents the amount (μg) of Na^+ and K^+ per gram of lyophilized tissues measured through the ICP-MS with five biological replicates. The comparisons for mineral accumulation were made between the contrasting NILs for the root tissue and shoot tissue independently. Asterisks above bars denote statistical significance for this differential mineral accumulation among the contrasting NILs assessed using Student's *t*-test, where *** $p \leq 0.001$. (F) Depicts the subcellular localization of *CaHKT1* protein in the *N. benthamiana* leaves. *CaHKT1*-YFP was observed to localise in the plasma membrane when the fusion construct was introduced into *Nicotiana benthamiana* leaves. Aquaporin-mCherry was used as a plasma membrane marker for confirmation. (G, H) *CaHKT1* demonstrates a Na^+ -preferential transporter activity in the *xenopus* oocyte cells. Oocytes expressing *CaHKT1* were subjected to voltage clamp experiments in bath solutions containing varying concentrations of Na^+ (G) and K^+ (H), where the shift of the reverse potential in the X-axis confirms the Na^+ ion selectivity.

CaHKT1-OE lines accumulated less Na^+ , maintained stable K^+ levels, and had a reduced Na^+/K^+ ratio compared to wild-type (WT) plants. In contrast, the roots of *CaHKT1*-OE lines showed higher Na^+ accumulation (Figure 4D–F). Furthermore, Corona green staining revealed that *CaHKT1*-OE roots efficiently sequestered Na^+ in the stellar region, preventing its entry into the transpiration stream, thereby protecting shoots from salt toxicity. Conversely, inefficient Na^+ exclusion by the WT plants resulted in shoot Na^+ toxicity that could have subsequently jeopardised reproductive events (Figure 4G). The findings confirm that the delineated *CaHKT1* protein functions as an Na^+ -preferential uniporter in chickpeas. The Na^+ exclusion behaviour observed in *CaHKT1*-OE lines aligns with that in salinity-tolerant NIL/accession (*ST-NIL^{HKT1}* and ICC 9942), explaining their enhanced salinity tolerance (Figure 3C–E; Figure 4D–G).

2.7 | Root-Specific Expression of *CaHKT1* Enhances Yield Endurance by Improving Reproductive Success of the Salt-Sensitive Chickpea Line

Yield endurance under salinity stress represents the ability of a plant to maintain stable crop yields despite saline soil conditions. It measures the resilience and adaptive potential of a plant to protect its growth and reproduction. Under salinity stress (4 dS/m), *CaHKT1*-OE lines showed less reduction in pod setting than the WT (ICC 6303), with comparable biomass, highlighting the role of *CaHKT1* in maintaining yield under saline conditions (Figure 5A–G). In addition to improving pod setting, *CaHKT1*-OE lines rescued the shrivelled seed phenotype observed in WT plants, resulting in higher seed weight under salinity stress (Figure 5B,C,F). The results demonstrate that yield is more significantly affected by salinity stress than by vegetative biomass.

As yield reflects reproductive success, the author examined the reproductive responses to understand the yield advantage of *CaHKT1*-OE lines under salinity stress. In WT plants, salinity stress caused a notable decrease in pollen viability, with distorted pollen grains, whereas *CaHKT1*-OE lines maintained viable pollen with an intact surface structure (Figure 5H,I). Pollen

dispersal was also impaired in the WT due to stunted stamen length; however, *CaHKT1*-OE lines resisted such stunting, enabling proper dispersal of viable pollen grains (Figure 5J,K). Scanning electron micrographs revealed shrivelled singular ovules in WT plants, whereas *CaHKT1*-OE lines exhibited multiple healthy ovules (Figure 5B,C). This explains the higher shelling percentage of *CaHKT1*-OE lines than that of the WT under salinity stress (Figure 5G). Thus, the final yield endurance of *CaHKT1*-OE lines under salinity stress was the product of numerous activities on diverse fronts in terms of reproduction.

2.8 | Ectopic Expression of *CaHKT1* Rescued Salt Sensitivity of *athkt1* Mutant

To validate the role of *CaHKT1* in salinity tolerance, a loss-of-function *athkt1 Arabidopsis* mutant was complemented with a *CaHKT1* construct driven by the native *AtHKT1* promoter. Under control conditions, WT, *athkt1*, and complemented lines showed similar growth rates. However, under salt stress, *athkt1* exhibited severe leaf scorching, whereas the WT and complemented lines displayed milder symptoms, indicating rescue of the salt hypersensitive phenotype (Figure 6A; Figure S3). Confocal imaging showed stronger CoroNa Green fluorescence in the roots of the WT and complemented lines, confirming better Na^+ exclusion than in *athkt1* (Figure 6B). The defective Na^+ exclusion in the mutant led to excess shoot Na^+ , triggering higher reactive oxygen species (ROS) levels, as evidenced by intense Nitroblue tetrazolium (NBT) staining, resulting in tissue damage (Figure 6C). In contrast, *CaHKT1* expression in *athkt1* reduced ROS accumulation and oxidative damage, and restored salt tolerance. The results confirm that *CaHKT1* enhances Na^+ exclusion and mitigates salinity-induced oxidative damage in *Arabidopsis*.

2.9 | *CaPHL7*, Positioned Within a Trans-QTL, Boosts Salt Tolerance by Activating Expression of *CaHKT1*

So far, we have described the mechanism by which *CaHKT1* confers salt stress tolerance in chickpea. However, the putative

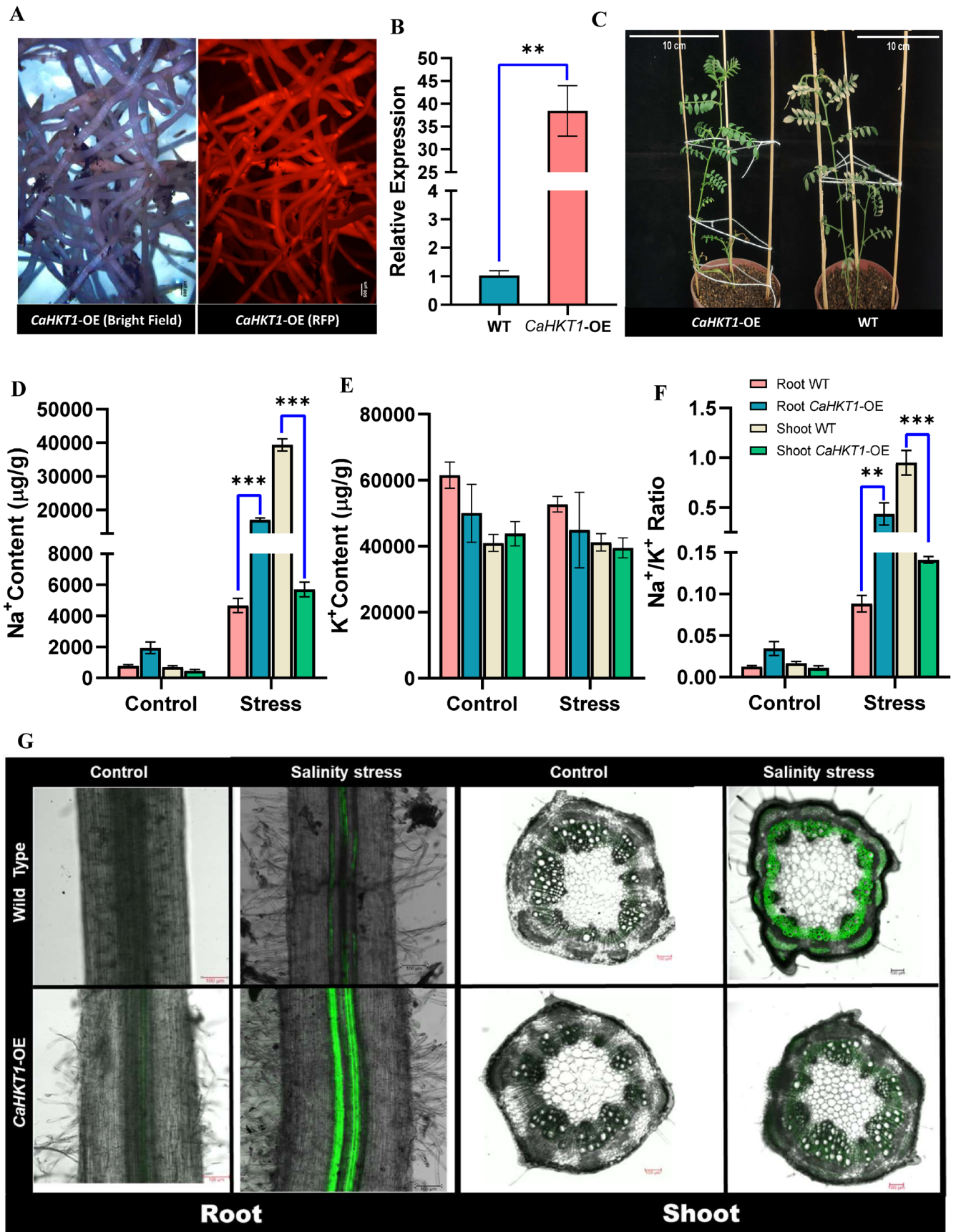


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FIGURE 4 | Root-specific overexpression of *CaHKT1* excludes Na^+ from the xylem sap when exposed to salinity stress condition. (A) Represents the hairy root transformation of ICC 6306 root for over-expressing *CaHKT1*, where the RFP used as a transformation marker for screening the positive transformants. (B) Confirms the overexpression of *CaHKT1* in the *CaHKT1*-OE lines by quantifying the relative amount of transcript compared to the wild-type (WT) (empty vector control) plants. The expression shown here are the cumulative mean of three different transformed plant compared to their empty vector control. Statistical significance is indicated by Student's *t*-test, where $**p \leq 0.01$ S.E. (Standard Error). (C) Demonstrates the phenotypic screening of *CaHKT1*-OE line with the WT plant under salinity stress condition, where the impact of stress is clearly visible in the scorched leaves of WT plants. (D–F) Represents the Na^+ content, K^+ content and Na^+/K^+ ratio respectively, in the root and shoot tissue of WT and *CaHKT1*-OE lines under control and salinity stress condition. Data represents the amount (μg) of Na^+ and K^+ per gram of lyophilized tissues measured through the ICP-MS with five biological replicates. Statistical significance is indicated by Student's *t*-test, where $***p \leq 0.001$ and $**p \leq 0.01$. (G) Represents the tissue-specific Na^+ accumulation in the root and shoot tissue of WT and *CaHKT1*-OE lines by sodium-specific Corona-green stain.

regulatory mechanism of *CaHKT1* remains unclear. Using an integrated genomics strategy, we delineated another gene, *CaPHL7*, harboured under the major QTL *CaqSYII.1*, which explains the high phenotypic variation in SYI (Figure 2). Hence, *CaPHL7* appears to be as crucial as *CaHKT1* in helping chickpea plants tolerate salt stress. A link emerged when we observed a co-expression pattern of *CaPHL7* with *CaHKT1* in both contrasting salinity-tolerant and -sensitive NILs, indicating its crucial role in *CaHKT1*-associated salinity stress response in chickpea (Figure 7A).

CaPHL7 is a 329 amino acid-long *Myb* family transcription factor featuring an HTH-Myb-DNA binding domain (46–106) and a c-terminus coiled-coiled dimerization domain (136–182) with a conserved 'LHEQLE' sequence motif, which classifies it into the MYB-CC family of transcription factors (Rubio et al. 2001). This protein family has been linked to phosphorus starvation responses in various plants, leading to the name PHR1-LIKE PROTEIN 7 (*PHL7*) (Rubio et al. 2001; Zhou et al. 2008; Wang et al. 2013). The *CaPHL7*-YFP fused protein was expressed transiently in *N. benthamiana* leaves, and the fluorescence pattern of the YFP-tagged *CaPHL7* showed a clear signal localised in the nucleus (Figure 7B). To investigate the regulatory role of *CaPHL7* in the salinity stress response, a potential binding site for *CaPHL7* was discovered in the 3-kb URR (promoter region) of *CaHKT1* using a plant reg map (Tian et al. 2020). To further validate the binding, a dual-luciferase assay was performed on *N. benthamiana* leaves. A high LUC/REN ratio (0.30) in the presence of *CaPHL7* compared to that in the control (0.08) suggested *CaPHL7*-mediated transcriptional activation of *CaHKT1* (Figure 7C,D). To further reinforce the finding, root-specific overexpression of *CaPHL7* was generated in the salinity stress-sensitive parental accession ICC 6306 by *A. rhizogenes*-mediated hairy root transformation (Figure 7E). Quantitative real-time PCR showed that the *CaHKT1* expression level was significantly higher in the *CaPHL7*-OE lines than in the WT, confirming *CaPHL7*-mediated transcriptional activation of *CaHKT1* (Figure 7F). Furthermore, comprehensive phenotyping of *CaPHL7*-OE lines, in contrast to the WT, showed tolerant agro-morphological and seed yield characteristics under salinity stress conditions, which supported our hypothesis, explaining the *CaPHL7*-induced *CaHKT1*-mediated salinity stress tolerance mechanism in chickpea (Figure 7G,H).

3 | Discussion

Sustainable yield and productivity are paramount for managing saline-affected areas and ensuring food security while optimising land use. The factors are particularly critical in arid and semi-arid regions, where salinity issues are pronounced and crops such as chickpea are commonly grown (Mehrotra et al. 2023). Understanding the genetic basis of tolerance and identifying molecular tags linked to salinity-responsive genes or QTLs are crucial for improving chickpea yield and productivity under salinity stress. However, salinity stress response is likely to be governed by multiple genes; therefore, dissecting the genetic basis of tolerance is a complex task. Furthermore, combining high yield and salinity tolerance is challenging, as tolerance often imposes metabolic costs, such as higher maintenance respiration and active ion transport, which can limit yield potential (Saxena et al. 1993; Gale and Zeroni 1985; Tal 1985). Salt-tolerant plants often prioritise survival over productivity. Therefore, molecular breeding strategies should be designed to ensure that identified genes or molecular tags also account for potential yield penalties. Hence, the degree of improvement by the delineated gene can be useful for demonstrating yield endurance under salinity stress conditions at the farm level.

To investigate yield endurance in chickpea under salinity stress, the authors employed a combined genomic strategy. Using SYI as a trait, GWAS identified two key loci on chromosomes 1 and 8. Subsequent regional association analysis, QTL mapping, fine mapping, and map-based cloning identified *CaHKT1* and *CaPHL7* as candidate genes that regulate yield under salinity stress. Although numerous HKT homologues with varying ion transport functions have been characterised across crops such as rice, wheat, maize, barley, grapevine, and *Arabidopsis*, the identification of functional alleles of *HKT1* that can resist the yield penalty under salinity stress remains largely unexplored (Horie et al. 2009; Zhang et al. 2018, 2023). *CaHKT1*, a class I Na^+ -selective transporter, was confirmed to localise to the plasma membrane and exhibited Na^+ -preferential transport in *Xenopus* oocytes. Phylogenetic analysis confirmed *CaHKT1* as an ortholog of type I HKT transporters found in wheat, rice, and *Arabidopsis*. Over the years, HKT1 genes involved in Na^+ uptake and xylem Na^+ retrieval have been identified across crops (Horie et al. 2009; Huang et al. 2020; Zhang et al. 2018). Genes harboured in salt-tolerant QTLs, such as the *OsHKT1;5*

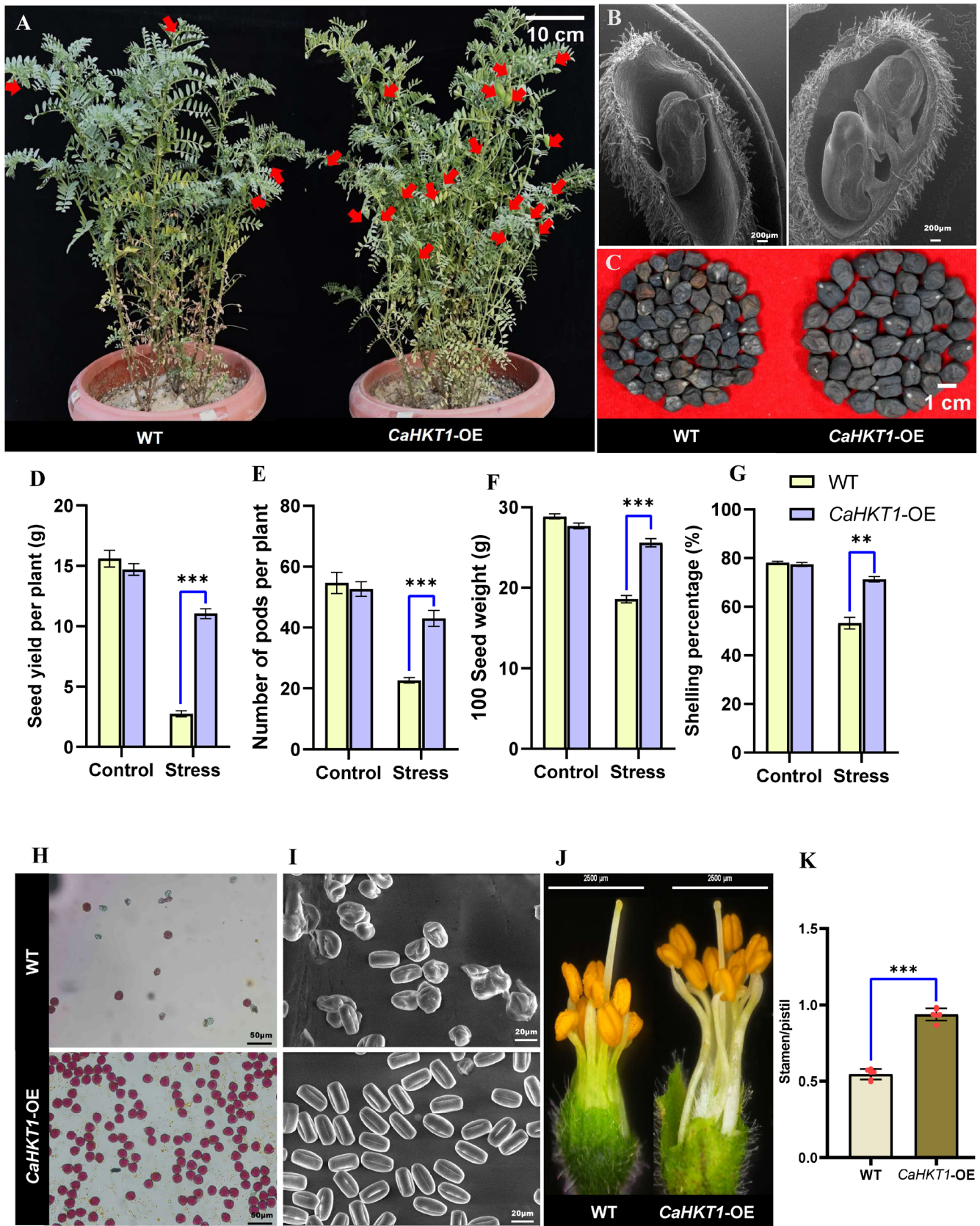


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FIGURE 5 | Phenotypic characterisation of CaHKT1-OE delineates higher reproductive success under salinity stress conditions compared to the wild-type (WT) at a stress of 4ds/m. (A) Depicts the significant loss of pod number in (WT) compared to CaHKT1-OE, whereas no discriminatory changes were witnessed in the vegetative growth. (B, C) Over-expression of *CaHKT1* rescues the shrivelled singular ovule phenotype of WT as witnessed from the scanning electron microscopy (SEM) (B) and ultimately improves the shrivelled seed phenotype of WT plant under salinity stress (C). (D–G) Represents diverse agronomic changes in seed yield (D), number of pods (E), seed weight (F) and shelling percentage (G) trait of CaHKT1-OE line compared to the WT under salinity stress condition. The data shown here are the cumulative mean of 3 different transformed plant compared to their empty vector control (WT). Statistical significance is indicated by Student's *t*-test, where *** $p \leq 0.001$ and ** $p \leq 0.01$. S.E. (Standard Error). (H, I) The pollen viability (H) and pollen morphology (I) of the WT and CaHKT1-OE lines were represented by Alexander staining and SEM, respectively. (J, K) The stunted stamen length of WT compared to the CaHKT1-OE line are depicted the pictorial representation (J) and quantifiable representation (K). The scoring was a representative measure of stamen/pistil ratio of six independent flower of WT and *CaHKT1*-OE lines under salinity stress condition. Statistical significance is indicated by Student's *t*-test, where *** $p \leq 0.001$.

gene under the major QTL *SKC1/SalTol* in rice (Ren et al. 2005; Thomson et al. 2010), *TaHKT1;5-D (Kna1)*, *TmHKT1;5-A (Nax2)*, and *TmHKT1;4-A2 (Nax1)* in wheat (Huang et al. 2006; Munns et al. 2012), *HKT1;5 (ZmNC1/ZmNC3)* in maize (Zhang et al. 2018, 2023), and QTL *NaE* in grapevine (Henderson et al. 2018) all encode HKT1-type Na^+ -selective transporters. These genes function to limit shoot Na^+ by retrieving it from the xylem (Horie et al. 2009; Henderson et al. 2018; Zhang et al. 2018). Although alleles such as *Nax2* have demonstrated potential in enhancing both salt tolerance and yield (Munns et al. 2012), the reproducibility of these outcomes remains uncertain, as the reported yield improvement was observed in only one out of three experimental blocks.

In the current study, *ST-NIL^{HKT1}*, *CaHKT1*-OE maintained lower shoot Na^+ compared to *SS-NIL^{HKT1}* and ICC 6306, driven by higher *CaHKT1* expression in roots. This explains the reduced ion toxicity and better yield performance in *ST-NIL^{HKT1}* and ICC 9942 than in their sensitive counterparts, *SS-NIL^{HKT1}* and ICC 6306. Moreover, the ability of *CaHKT1* to functionally complement the *Arabidopsis athkt1* mutant reinforces the evolutionarily conserved role of HKT1 transporters in ion homeostasis and salt stress adaptation, consistent with previous reports across multiple crop species (Rus et al. 2004; Møller et al. 2009).

A promising strategy for HKT1-based crop improvement is to enhance its expression, as several salt-tolerant genotypes consistently show higher root-level HKT1 transcription than sensitive genotypes (Hazzouri et al. 2018; Shohan et al. 2019; Wang et al. 2024; Song et al. 2024). In the present study, RT-PCR confirmed root-preferential expression of *CaHKT1* under salinity stress in *ST-NIL^{HKT1}*, *SS-NIL^{HKT1}*, and their parental lines. Since constitutive *HKT1* overexpression has previously failed to enhance salt tolerance due to its requirement for cell-type-specific expression (Møller et al. 2009; Plett et al. 2010; Ali et al. 2012), we instead developed root-specific overexpression (OE) lines of *CaHKT1* in the ICC 6306 background to achieve targeted expression and functional validation. The lines restored salt sensitivity, displayed lower shoot Na^+ , stable K^+ levels, and a reduced Na^+/K^+ ratio. Na^+ was effectively sequestered in root stellar tissues of the OE line, limiting translocation to shoots and preventing toxicity. This highlights the fact that *CaHKT1* expression levels directly influence Na^+ exclusion efficiency, thereby determining salt tolerance, particularly shielding the reproductive events.

Despite a decade of fundamental research on the well-established role of HKT1 in Na^+ regulation, its contribution to yield stability under field conditions remains unclear and difficult to reproduce (Munns et al. 2012; Zhang et al. 2018; Imran et al. 2019; Liu et al. 2024). Indeed, many authors have questioned the long-term value of *HKT1* as a breeding target, noting that its impact may diminish as global salinity intensifies and that its capacity to sequester Na^+ in root tissues is inherently limited (Liu et al. 2020; Shabala et al. 2025). In such a context, *HKT1*-mediated Na^+ exclusion should not be viewed as a universal solution, particularly under very high salinity levels, where plants must increasingly rely on osmotic adjustment and organic osmolyte accumulation, often at significant carbon cost (Zhu, 2016; Assaha et al., 2017; Wu et al., 2018). However, the *HKT1* function remains highly relevant within the low-to-moderate salinity range, which encompasses a substantial portion of the world's salt-affected agricultural land. Even moderate salinity, when sustained across the life cycle, can impose substantial cumulative yield losses, and the ability of *HKT1* to mitigate these penalties provides meaningful agronomic and economic benefit. This point is particularly critical for chickpea, which is one of the most salt-sensitive legumes, where even a low-to-moderate level of salinity causes significant yield reduction (Lauter and Munns 1987; Flowers et al. 2010; Dua 1992; Vadez et al. 2007; Serraj et al. 2004; Turner et al. 2013). In this context, the identification of superior allelic variants of *CaHKT1* and its regulator *CaPHL7* offers a valuable opportunity to enhance yield stability, extend chickpea cultivation into marginal soils, and contribute to sustainable pulse production and food security. Although *CaHKT1* is not a silver bullet for high-salinity tolerance, it plays a critical role in mitigating yield penalties under moderate salinity, a range highly relevant to chickpea cultivation.

Salinity stress in chickpea is known to critically affect the reproductive stage by impairing stigma viability, pollen germination, and pollen tube growth (Samineni et al. 2011). In our study, *CaHKT1*-OE lines exhibited improved seed yield under saline conditions, which is associated with higher pollen viability, optimal stamen elongation, successful fertilisation, and superior embryo development, leading to increased shelling percentage and final yield. These findings elucidate the yield resilience seen in *ST-NIL^{HKT1}* and ICC 9942, closely resembling the performance of the *CaHKT1*-OE line under salinity stress. Similar reproductive phenotypes have been observed in *Arabidopsis*, where loss of *AtHKT1;1* causes impaired stamen elongation and male sterility, whereas tissue-specific *AtHKT1;1* expression

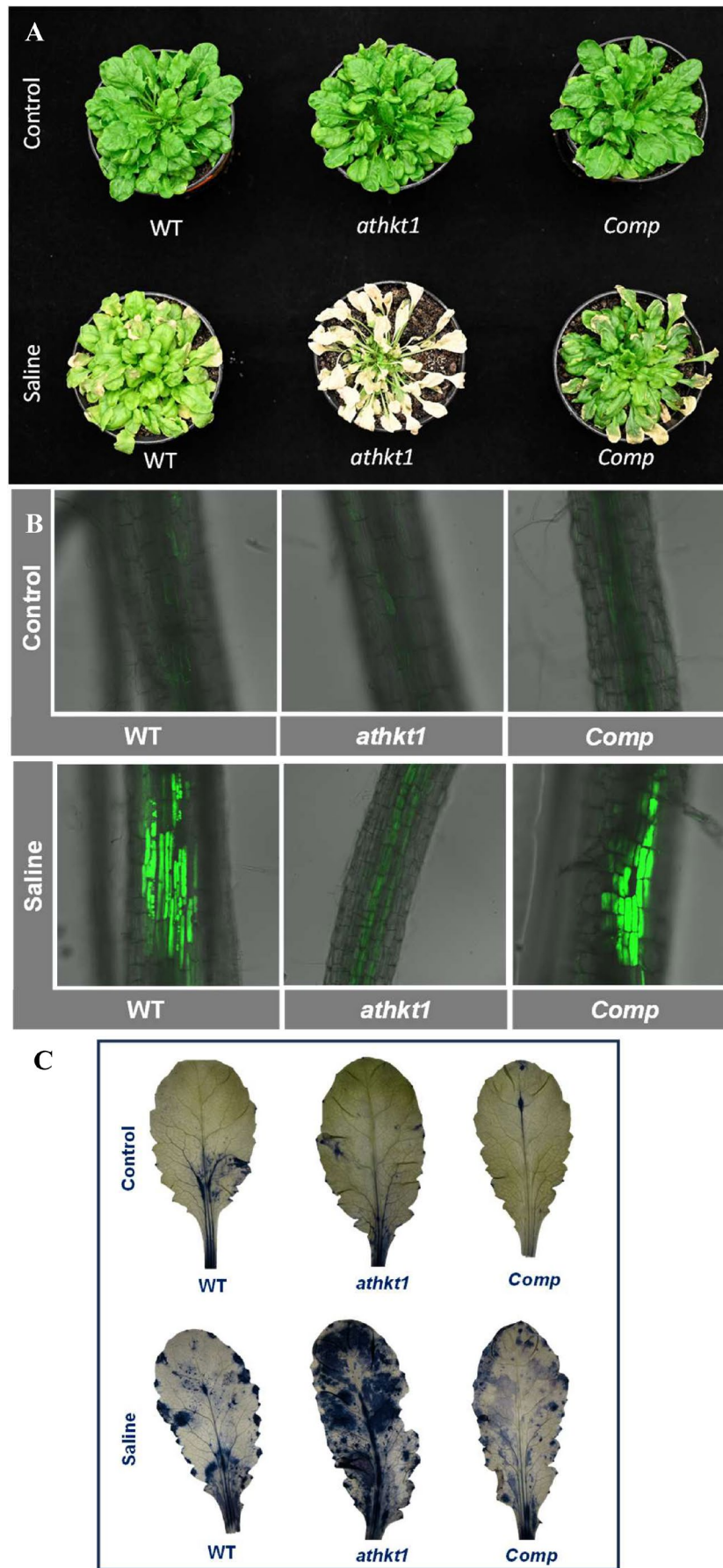


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FIGURE 6 | *CaHKT1* can rescue the salinity sensitive phenotype of *athkt1* mutant. (A) Represents the phenotypic changes of WT, *athkt1* and complemented *Arabidopsis* line under control and salinity stress conditions. The complemented line represents restoration of *pAtHKT1::CaHKT1* in the mutant *athkt1* background. (B) Represents the root-specific Na^+ accumulation in the WT, *athkt1* and complemented *Arabidopsis* line under control and salinity stress condition. The GFP signal intensity captured by the confocal microscopy with Na^+ specific corona green stain corresponds to the amount of Na^+ accumulating in the root tissues of the mentioned *Arabidopsis* lines. (C) Represents the differential ROS accumulation in the WT, *athkt1* and complemented *Arabidopsis* lines under normal and salinity stress conditions. The differential NBT precipitates in the leaf tissues of the mentioned lines correspond to the amount of ROS accumulated in the leaf tissues which is proportional to the amount of stress the above ground part experiencing due to the salinity stress.

enhances seed yield by 1.5-fold under salt stress (Uchiyama et al. 2023). Furthermore, the reproductive success observed in the *CaHKT1*-OE lines, despite no vegetative differences compared to the WT, suggests that reproductive tolerance would not have been effectively captured through vegetative-stage phenotyping under salinity stress. The results further validate the effectiveness of using SYI as a measurable trait to assess salinity stress tolerance, highlighting its translational significance in maintaining stable yield under salinity stress in chickpea.

Because HKT1 function depends on tissue-specific expression, understanding its upstream regulation is essential. Although the regulation of transporter families, such as NRTs, is well characterised (Vidal et al. 2020), HKT1 regulation remains partially understood. Known upstream regulators include TaSPL6-D in wheat, AtABI4 in *Arabidopsis*, and OsSUVH7–OsBAG4–OsMYB106 and OsSDG721 in rice, which modulate HKT1 via transcriptional or epigenetic mechanisms (Kronzucker and Britto 2011; Mäser, Hosoo, et al. 2002; Sunarpi et al. 2005; Shkolnik-Inbar et al. 2013; Wang, Nan, et al. 2020; Liu et al. 2021). In the present study, we identified a novel regulator, *CaPHL7*, within a trans-QTL region that controls *CaHKT1* expression. Although the role of *PHL7* in salt tolerance has recently been reported in rice (Yang et al. 2024), the precise mechanisms and its impact on yield parameters remain unresolved. In the present study, promoter analysis and dual-luciferase assays confirmed that *CaPHL7* directly transactivates *CaHKT1*. Furthermore, *CaPHL7* overexpression resulted in increased *CaHKT1* expression, which is consistent with salinity-induced expression data from *NIL^{PHL7}* and parental accessions. Additionally, *CaPHL7*-OE lines showed a yield stability proportional to that of *CaHKT1*-OE, confirming a trans-QTL regulatory model in which *CaPHL7* modulates *CaHKT1* expression and defines the degree of salinity tolerance in chickpea.

This study presents a pioneering example of a trans-QTL interaction in chickpea, where the superior haplotype in ST accessions elevates *CaPHL7* expression, which in turn transactivates *CaHKT1*, thus regulates Na^+ exclusion, and ensures reproductive success and yield stability under salt stress (Figure 8). This aligns with previous evidence that trans-QTLs can regulate distant gene expression and influence key traits across crops (Li et al. 2018, 2024; Hartanto et al. 2022; Abraham and Croll 2023). A well-known example is the *FRI*-*FLC* interaction in *Arabidopsis thaliana*, in which *FRIGIDA* (*FRI*), located on chromosome 4, activates the *FLOWERING LOCUS C* (*FLC*) on chromosome 5 to repress flowering. These two genes were narrowed down from two different QTLs that control flowering time in *Arabidopsis*. Variations in the loci underlie the natural differences in flowering time by controlling the

vegetative-to-reproductive transition, a critical adaptive trait in response to seasonal cues (Shindo et al. 2005; Kover et al. 2009). With few functional trans-QTL examples reported in plants, our integrated approach offers a valuable framework for unravelling complex regulatory networks. The previously unexplored regulatory link revealed in this study suggests potential crosstalk between phosphate signalling and salinity tolerance, opening new avenues for research on stress adaptation in chickpea and other crops. Furthermore, the delineated superior alleles will serve as functional targets for precise genome editing, and their tightly linked markers will enable the precise introgression of these alleles into elite cultivars. This will pave the way for the development of high-yielding, climate-smart chickpea varieties with enhanced salinity tolerance and minimal yield penalties.

4 | Experimental Procedures

4.1 | Constitution and Phenotyping of a Chickpea Diversity Panel for Salinity Stress Tolerance Traits

A chickpea diversity panel representing over 90% of global core germplasm (1956 accessions; Table S1), including 189 *desi* and 102 *kabuli* accessions from 30 ecogeographic regions, was used for GWAS. The panel was phenotyped under an open-air rain-out shelter at ICRISAT, Hyderabad, India, in bottom-sealed pots filled with 7.5 kg soil supplemented with DAP (Diammonium Phosphate; 300 mg/kg). Salinity stress was imposed by applying an 80 mM NaCl solution at the time of sowing, in a volume sufficient to saturate the soil to its field capacity. This was followed by two irrigations with tap water throughout its growing period. This approach was adapted from the well-established protocol by Vadez et al. (2007), widely used at ICRISAT for large-scale chickpea salinity screening. Due to the soil's inherent buffering capacity, this treatment effectively produced a net rhizospheric salinity level of approximately 4 dS/m. This effective rhizospheric electrical conductivity (EC) was measured using a FieldScout EC450 meter (Spectrum Technologies Inc., Aurora, IL, USA), equipped with a soil-penetrating, direct-insertion electrode designed to record real-time EC values directly within the rhizosphere. The trial followed a randomised block design, and seed yield (g/pot) was recorded for 2 years. The SYI was calculated for each accession (Raman et al. 2012). Statistical analyses (mean, standard deviation, coefficient of variation, frequency distribution, and Pearson's R) were calculated to assess SYI variation (Bajaj, Saxena, et al. 2015; Upadhyaya et al. 2015). Analysis of Variance (ANOVA) was used to estimate the genotype (G), environment (E), and G × E interactions (Srivastava et al. 2017). Broad-sense heritability (H^2) was calculated using: $H^2 = \sigma^2_g / [\sigma^2_g + \sigma^2_{ge}/n + \sigma^2_e/nr]$, with $n = 2$ environments, $r = 3$ replicates,

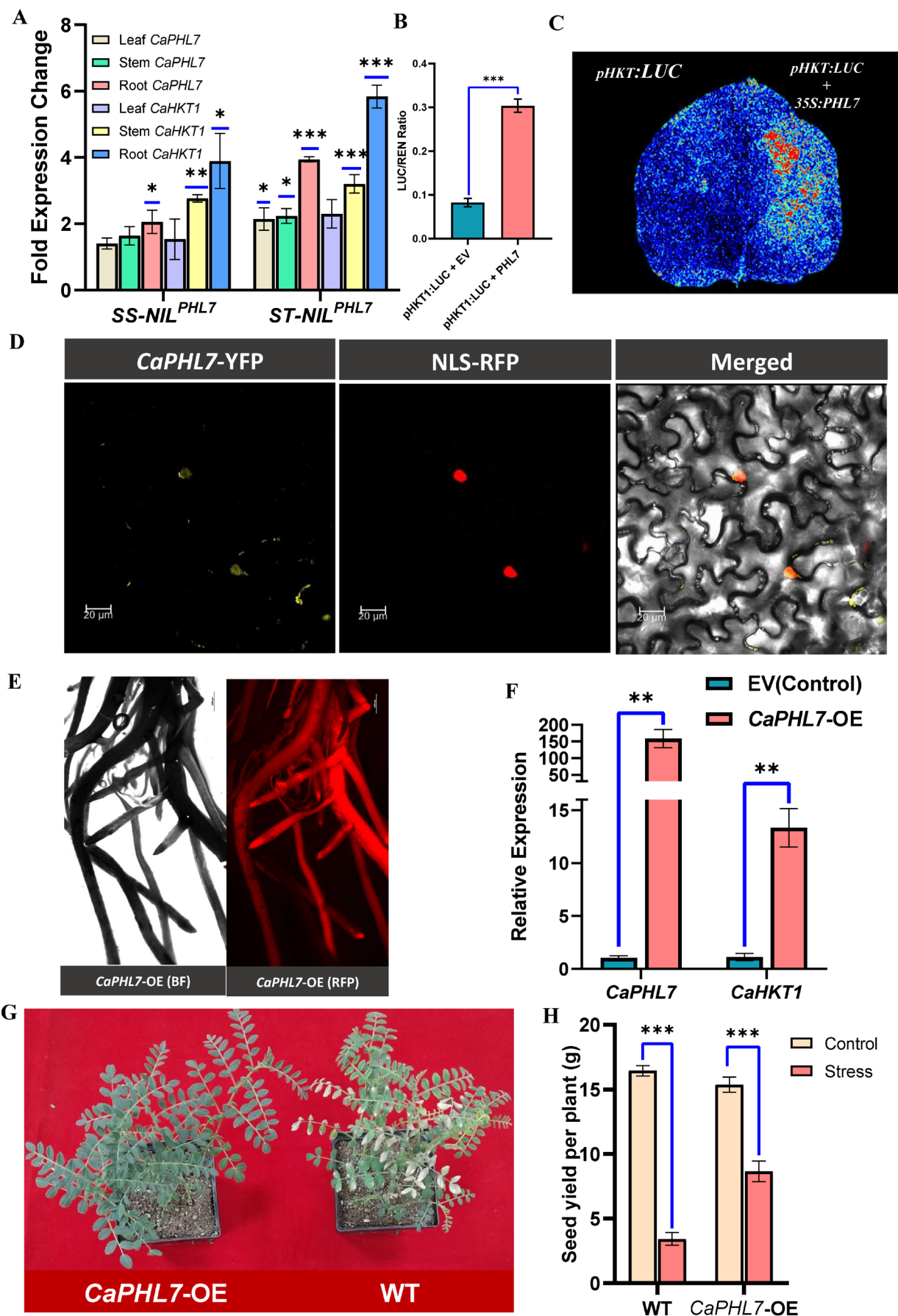


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FIGURE 7 | CaPHL7 transcriptionally activates the *CaHKT1* to relay salinity stress tolerance in chickpea. (A) Relative expression levels of *CaPHL7* and its co-expression with *CaHKT1* in root, stem, and leaf tissues of contrasting salinity-responsive NILs (*SS-NIL^{PHL7}*, *ST-NIL^{PHL7}*). Each bar represents the fold change in gene expression under salinity stress relative to its corresponding control condition, in the same tissue and genotype. Data represent the means $\pm$ SE with three biological replicates. Asterisks above bars denote statistical significance of fold expression differences between salinity-stressed and control conditions for the same tissue and genotype, assessed using Student's *t*-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). (B) Represents the relative LUC/REN activity measured in *Nicotiana benthamiana* leaves co-infiltrated with the reporter construct (*ProCaHKT:FireflyLuc*) along with the specified effector (EV/2X-35S:CaPHL7) constructs. The *Renilla luciferase* (REN) from the 35S construct was used for normalisation. Data represent the means $\pm$ SE with three biological replicates and the statistical significance is indicated by Student's *t*-test, with *** $p \leq 0.001$. (C) qualitative depiction of the dual luciferase assay from the *N. benthamiana* leaves captured through the CCD imaging apparatus. (D) Depicts the sub-cellular localization of CaPHL7 protein in the *N. benthamiana* leaves. CaPHL7-YFP was observed to localise in the nucleus when the fusion construct was introduced into *Nicotiana benthamiana* leaves. NLS-RFP was used as a nuclear marker for confirmation. (E) Represents the hairy root transformation of ICC 6306 root for overexpressing *CaPHL7*, where the RFP used as a transformation marker for screening the positive transformants. (F) Confirms the overexpression of *CaPHL7* in the CaPHL7-OE lines by quantifying the relative amount of transcript compared to the wild-type (WT) (empty vector control) plants. Subsequently, the CaPHL7-OE line also demonstrates the elevated expression of *CaHKT1* compared to WT control. The expression shown here is the cumulative mean of 3 different transformed plant compared to their empty vector control. Statistical significance is indicated by Student's *t*-test, where ** $p \leq 0.01$ S.E. (Standard Error). (G) Demonstrates the phenotypic screening of CaPHL7-OE line with the WT plant under salinity stress condition, where the impact of stress is clearly visible in the scorched leaves of WT plants. (H) Represents the differential seed yield of CaPHL7-OE and WT lines under control and salinity stress condition.

and σ^2_g , σ^2_{ge} , and σ^2_e representing genetic, interaction, and error variances.

4.2 | Generation and Phenotyping of a Chickpea Mapping Population for Salinity Stress Tolerance Traits

Multi-season field phenotyping identified two contrasting desi genotypes: ICC 9942 (salinity tolerant, SYI 0.88) from the USSR and ICC 6306 (salinity sensitive, SYI 0.55) from India. These were inter-crossed to develop a 216-line F_{10} RIL population, which was phenotyped for SYI using the same methods as the diversity panel.

4.3 | Mining, Genotyping and Annotation of Genome-Wide SNPs

For GBS, high-quality genomic DNA was extracted from 291 diversity panel accessions, 216 RILs, and parental lines using a DNeasy 96 Plant Kit (QIAGEN, USA). DNA was digested with ApeKI, ligated with barcoded adaptors, used to construct 96-plex libraries, and sequenced on an Illumina HiSeq2000 (Illumina Inc., San Diego, CA, USA) to generate 100-bp paired-end reads. Quality filtering (Phred > 30) of the demultiplexed reads was performed using NGS QC Toolkit v2.3 (Patel and Jain 2012). Reads were aligned to the chickpea reference genome v1.0 (Varshney et al. 2013) using Burrow Wheeler Aligner (Li and Durbin 2010), and SNPs were identified using the STACKS v1.0 GBS pipeline (Kujur, Bajaj, Upadhyaya, Das, et al. 2015; Kujur, Bajaj, Upadhyaya, et al. 2015; Kujur, Upadhyaya, et al. 2015). SNP annotation was performed using snpEff v4.3p (Cingolani et al. 2012), and genomic distribution was visualised using density plots.

4.4 | Assessing Molecular Diversity and Population Genetic Structure

SNP genotyping data mapped across eight chickpea chromosomes were used to analyse the genetic diversity, phylogeny, and

population structure of 291 chickpea accessions in a diversity panel. The phylogenetic relationship was determined through the NJ clustering method in TASSEL 5.0 (Bradbury et al. 2007). To assess population stratification, ADMIXTURE Version 1.3.0 (<https://dalexander.github.io/admixture/download.html>) was used with a range of 1–9 subpopulations, with 5-fold cross validation, and subsequently visualised using a stacked bar plot.

4.5 | Genome-Wide Association Study

A GWAS was conducted on 291 chickpea accessions using GAPIT v3 (Lipka et al. 2012) to identify SNP associations with SYI by applying the BLINK model (Huang et al. 2019), which incorporates genome-wide LD information. PCA and kinship matrices were used to account for population structure and genetic relatedness. Significant associations were illustrated using Manhattan plots, whereas Q-Q plots assessed the accountability of the models in addressing family relatedness and population structure. To control false positives, Benjamini-Hochberg FDR correction (cut-off ≤ 0.05) was applied (Kujur, Bajaj, Upadhyaya, et al. 2015), and FDR-adjusted P-values were used to identify significant SNPs. The phenotypic variation explained (PVE) for each SNP associated with SYI was also calculated.

4.6 | Genetic Linkage Map Construction and High-Resolution QTL Mapping

GBS-derived SNPs (82247) polymorphic between ICC 9942 (ST) and ICC 6306 (SS) were genotyped in 216 RILs using the Sequenom MALDI-TOF MassARRAY (Saxena Bajaj, Das, et al. 2014). A genetic linkage map was constructed with JoinMap 4 using the Kosambi function and an LOD threshold of 4.0–10.0 (Stam 1993; Kujur, Upadhyaya, et al. 2015). SNPs were grouped into LG1–LG8 based on genetic (cM) and physical (bp) positions and visualised using MapChart v2.2 (Voorrips 2002). QTL mapping was performed using composite interval mapping in MapQTL 6 (LOD > 5.0; 1000 permutations at $p < 0.05$), following Das et al. (2015) and Kujur,

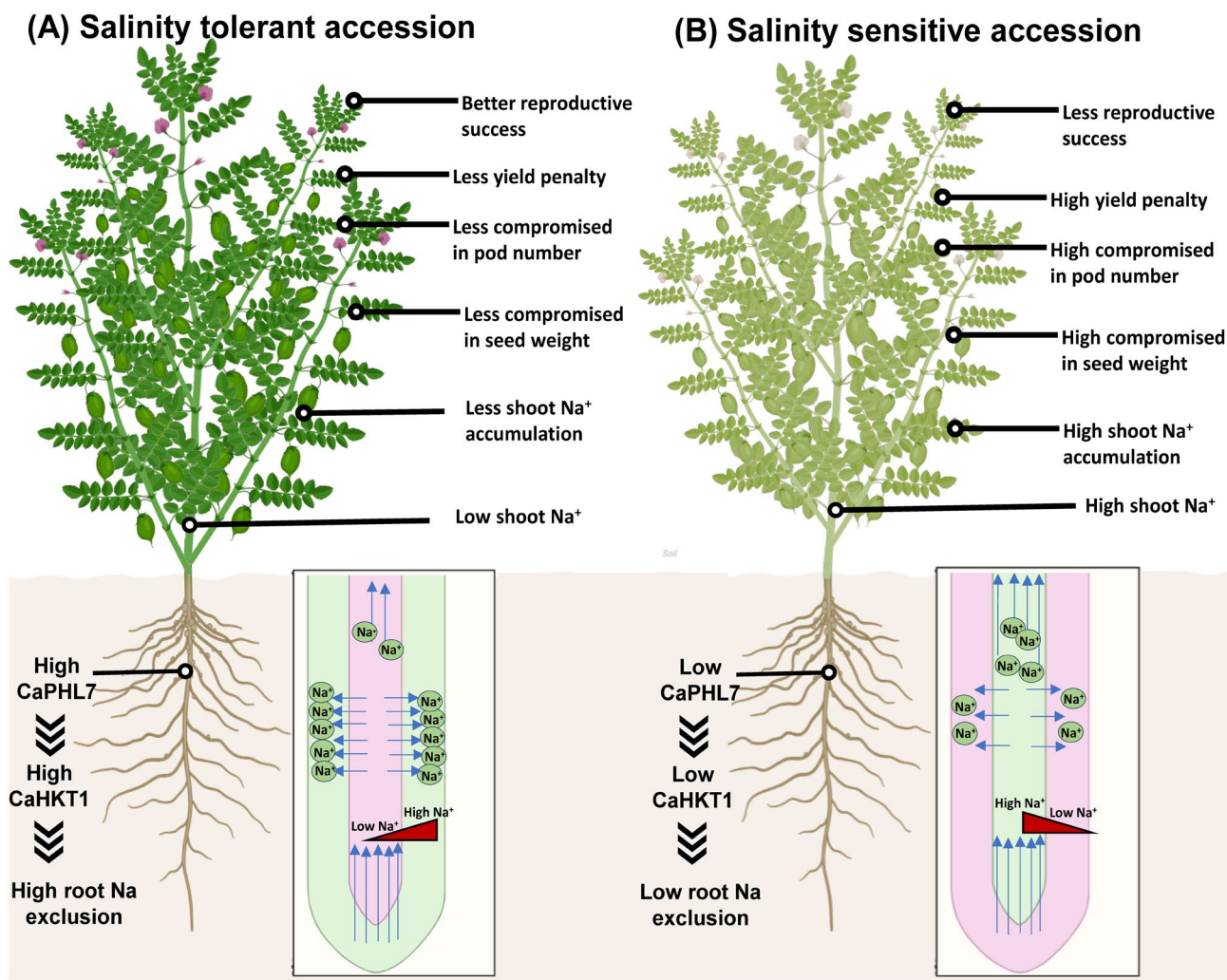


FIGURE 8 | A schematic illustration of the transcriptional regulatory function of *CaPHL7* and *CaHKT1* relaying yield endurance in chickpea under salinity stress. The diagram depicts differential induction of *CaPHL7* and *CaHKT1* in the contrasting accessions for salinity stress response in chickpea. (A) The salinity-tolerant (ST)-accession/ST-NIL with their superior gene haplotype, demonstrates an elevated expression of *CaPHL7* that subsequently trans-activate the *CaHKT1* leading to efficient Na^+ exclusion in the root tissue. This event resulted in maintaining an ideal sodium concentration in the transpiration stream and keeping the above ground part protected from the devastating impact of salinity stress. The resultant outcome is evident from the enduring reproductive success that ultimately reflects in diverse yield contributing traits. (B) Contrary to that, the salinity-sensitive (SS)-accession/SS-NIL with their inferior haplotype, demonstrate a minimal fold expression change in *CaPHL7* that hardly manage to trans-activate the *CaHKT1* leading to a less efficient Na^+ exclusion in the root tissue. This incompetency of the sensitive accession/NIL fails to maintain an ideal sodium concentration in the transpiration stream which lead to Na^+ toxicity in the above ground part demonstrating a devastating impact of salinity stress. This impact is subsequently reflected in the reproductive failure leading to yield penalty.

Upadhyaya, et al. (2015). The optimal QTL model was identified using stepwise forward selection and backward elimination strategies, and the PVE% of each QTL on the SYI was assessed using ANOVA (Bajaj, Upadhyaya, et al. 2015; Malik et al. 2023). We determined the confidence interval (CI) for each significant major QTL peak using ± 1 -LOD support intervals (95% CI). Reaction norm plots for major SYI QTLs across years were created with the R 'rxnNorm' function.

4.7 | Fine-Mapping and Map-Based Cloning

Two key QTLs, *CaqSY11.1* (10.46kb) and *CaqSY18.1* (6.7kb), linked to salinity tolerance (SYI), were selected for

fine-mapping. BC_4F_5 NILs (SS-NIL and ST-NIL) for each QTL were developed via marker-assisted foreground and background selection by introgressing QTLs from SS (low SYI) and ST (high SYI) RILs into their reciprocal recurrent parental backgrounds. These contrasting NILs were inter-crossed to generate 311 (ST-NIL^{*CaqSY11.1*} $\times$ SS-NIL^{*CaqSY11.1*}) and 331 (ST-NIL^{*CaqSY18.1*} $\times$ SS-NIL^{*CaqSY18.1*}) F_2 individuals for subsequent fine mapping and progeny testing. To fine-map *CaqSY11.1* and *CaqSY18.1*, genomic DNA from parental lines (ICC 6306 and ICC 9942) and selected homozygous F_2 individuals (311 for *CaqSY11.1*, 331 for *CaqSY18.1*) with contrasting SYI were sequenced using amplicon resequencing (Saxena Bajaj, Kujur, et al. 2014). Parental polymorphic SNPs within the 10.46kb and 6.7kb QTL regions were genotyped across the respective F_2 s using Sequenom

MALDI-TOF MassARRAY. The resulting genotypic data were integrated with SYI phenotypes from both the F_2 populations and the 291-accession diversity panel for QTL fine-mapping and association analysis, respectively.

Progeny analysis was performed using homozygous recombinant and non-recombinant progenies from *ST-NIL* $\times$ *SS-NIL* crosses for *CaqSYI1.1* and *CaqSYI8.1*. Selection was based on SYI trait contrast and recombination at SNPs flanking the QTLs and candidate genes (*CaPHL7*, *CaHKT1*). These lines were phenotyped for SYI across environments using a randomised complete block design (RCBD), and SYI differences were evaluated using a one-tailed *t*-test. NILs carrying SS or ST haplotypes for *CaPHL7* and *CaHKT1* were developed by marker-assisted selection: foreground selection using SNP linked to QTLs/associated genes and background selection with 1536 parental polymorphic SNPs across eight chromosomes. Large-scale field phenotyping of NILs and backcross progenies followed the same SYI assessment strategy as mentioned for the diversity panel.

4.8 | Genome-Wide Identification and Genomic Constitution of *HKT*

A genome-wide identification of HKT transporter genes in the chickpea reference genome (Varshney et al. 2013) was performed using HMMER (<http://hmmer.org>) with a custom-built hidden Markov model (HMM) constructed from amino acid sequences of 13 diverse plant species, including model dicots (*Arabidopsis thaliana*), monocots (*Oryza sativa* and *Triticum aestivum*), and other related legumes, such as *Medicago truncatula*, *Lotus japonicus*, *Trifolium pratense*, *Glycine max*, *Arachis hypogaea*, *Cajanus cajan*, *Vigna angularis*, *Vigna radiata*, *Lupinus angustifolius*, and *Phaseolus vulgaris*. An *E*-value threshold of $1e-05$ was applied to retrieve candidate sequences. The functional integrity of retrieved sequences was validated by domain analysis using INTERPRO (<https://www.ebi.ac.uk/interpro>), confirming their classification as HKT transporters.

4.9 | Phylogeny of *CaHKT1*

To understand the evolutionary relationship of *CaHKT1*, its protein sequence was subjected to reciprocal BLAST analysis against representative orthologs from selected model species and legumes. The orthologous sequences were aligned using CLUSTALW with the BLOSUM62 matrix (<https://www.genome.jp/tools-bin/clustalw>). The presence of conserved HKT domains in all orthologs was confirmed through INTERPRO. A neighbour-joining phylogenetic tree was constructed with 1000 bootstrap replicates using MEGA11 (Kumar et al. 2016) to assess the relatedness of *CaHKT1* to other HKT family members.

4.10 | Control Environment Stress Treatment and Differential Expression Profiling

Contrasting chickpea genotypes, ICC 9942 and ICC 6306, were grown in pots containing a 1:1 mixture of agropit and soilrite

under controlled conditions in a Phytotron chamber (Conviron CMP6050). After 21 days, salinity stress (100 mM NaCl) was applied to reach field capacity, while the controls received water. Rhizosphere salinity was maintained at 4 dS/m using a FieldScout EC450 meter (Spectrum Technologies Inc., Aurora, IL, USA) to ensure consistent stress. Two parallel experiments were conducted: a short-term (24-h) treatment to assess *CaHKT1* and *CaPHL7* expression and a long-term experiment for phenotypic evaluation.

The differential expression of *CaHKT1* and *CaPHL7* in ST and SS genotypes/NILs was analysed using qRT-PCR (Malik et al. 2020). RNA from root/shoot tissues was extracted using the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA), treated with DNase I, and reverse-transcribed using the Verso cDNA Kit (Thermo Scientific, Waltham, MA, USA). Gene amplification was performed using diluted cDNA, gene-specific primers (Table S2), and iTaq SYBR Green Supermix on a BIORAD CFX96 system (BioRad, Hercules, CA, USA). Actin was used as an internal control (Garg et al. 2010), and relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Bajaj, Upadhyaya, et al. 2015; Malik et al. 2020).

4.11 | Subcellular Localization

The coding sequences of *CaHKT1* and *CaPHL7* were cloned into the gateway vector pSITE-3CA (Chakrabarty et al. 2007) to create YFP fusion constructs (*Pro35S:CaHKT1-YFP* and *Pro35S:CaPHL7-YFP*), with *Pro35S:eYFP* as the control. These constructs were transformed into *Agrobacterium* strain EHA109 and infiltrated into *N. benthamiana* leaves. Plants were incubated overnight at 24°C in the dark and then subjected to a 16-h light/8-h dark cycle. After 48 h, the YFP signals were visualised using a Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany) with a 20 $\times$ optical zoom.

4.12 | Vector Construction and Chickpea Hairy Root Transformation

Full-length coding sequences of *CaHKT1* and *CaPHL7* were amplified from the salt-tolerant chickpea genotype ICC 92944 using gene-specific primers (Table S2) and cloned into the pENTR vector using the D-TOPO Cloning Kit (Thermo Fisher, USA). The inserts were recombined upstream of the RFP marker under the control of the ubiquitin promoter in the gateway-based vector, pUB-RR-cGFP (Kryvoruchko et al. 2016). The resulting constructs (*pUBI:CaHKT1.1-RFP* and *pUBI:CaPHL7-RFP*) and the empty vector were transformed into *Agrobacterium rhizogenes* (ARQual) (Boisson-Dernier et al. 2001; Singh et al. 2020). Hairy root transformation was performed using the salinity-sensitive chickpea genotype ICC 6306. Sterilised seedlings were trimmed at 1 cm root length, coated with ARQual culture, and incubated on Fahraeus media at 22°C (14-h light/10-h dark) for 12–14 days. Transgenic roots were identified using RFP fluorescence under a stereomicroscope.

4.13 | Dual Luciferase Reporter Effector Assay

The cis-regulatory region of *CaHKT1* from the salt-tolerant chickpea genotype ICC 92944 was cloned into the pENTR vector

and transferred into the destination vector p635nRRF, containing 35S: RENILLA (REN) (normaliser) and ProCaHKT1: Firefly Luciferase (LUC: reporter), using Gateway LR Clonase II (Kumar et al. 2018). The CaPHL7 coding sequence was cloned into pMDC32, using a dual 35S promoter, to generate an effector construct. Reporter and effector constructs were co-infiltrated into *N. benthamiana* leaves via *Agrobacterium*-mediated transformation. After 48 h, LUC and REN activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) on a POLARstar Omega plate reader (BMG Labtech, Ortenberg, Germany), and LUC activity was normalised to REN. The infiltrated leaves were visualised under low light using a CCD imaging system (Bio-Rad).

4.14 | Estimation of Tissue Mineral Content

Unstressed control and salinity stress-imposed chickpea root and shoot tissues were harvested with 5 biological replicates and lyophilized for 3 days. Equivalent weights of the lyophilized and homogenised tissues were digested with 70% nitric acid at 180°C using a microwave digestion system. The digested samples were used to measure the mineral (Na^+ , K^+) ion concentrations using ICP-MS (ICP-MS 7800; Agilent Technologies, Santa Clara, CA, USA).

4.15 | Localization of Na^+ Ions Through Fluorescence Probe

Tissue-specific Na^+ accumulation in root and shoot tissues of control and salt-stressed chickpea plants was visualised using the Na^+ -specific dye CoroNa-Green AM and a Leica TCS-SP8 confocal microscope (Leica TCS-SP8, Leica). Thin hand-cut sections were incubated for 3 h in 20 μM CoroNa-Green AM in 0.02% (w/v) pluronic acid, following Marriboina et al. (2017). The samples were then imaged with excitation at 492 nm and emission at 516 nm.

4.16 | Functional Analysis of *CaHKT1* in *Xenopus laevis* Oocytes Using Two-Electrode Voltage Clamp-Based Electrophysiological Assay

The oocytes were surgically isolated from the mature female *X. laevis* and defolliculated enzymatically and further stored at 16°C–18°C in Barth's buffer. The protein-coding sequence (CDS) of *CaHKT1* was amplified using forward and reverse primers and cloned into the oocyte-specific expression vector, pGEMHE. Subsequently, capped mRNA (cRNA) coding for *CaHKT1* was synthesised using mMESAGG EmMACHINE T7 Transcription Kit (Thermo Fisher Scientific) and made up to a final concentration of 1.0 $\mu\text{g}/\mu\text{L}$ prior to storage at –80°C. To perform two-electrode voltage clamp-based electrophysiological analysis, healthy *X. laevis* oocytes (stage V/VI) were injected individually with either 20 nL (20 ng) of cRNA or an equal volume of RNA-free water as the control. The oocytes were then cultured in a bath buffer (serving as a recording solution) containing variable concentrations of Na^+ / K^+ gluconate salt (as a glutamate salt) and incubated with glass micropipette electrodes filled with 3M KCl. The activities of the oocytes were documented

for 36–48 h after injection using the 2-electrode voltage clamp method at 25°C, and finally, data analysis was performed with a Clamp500 amplifier (Molecular Devices, San Jose, CA, USA) and Clampex software (Molecular Devices) as described earlier (Liu et al. 2001; Horie et al. 2007; Cao et al. 2019). For this, a holding membrane potential of 60 mV was used and further oocytes were clamped stepwise from +60 to –180 mV with 30 mV decrements for 600–800 ms to measure the ion (Na^+ / K^+) selectivity and transport activities of a salinity stress-responsive gene, *CaHKT1*.

4.17 | Assessing Pollen Viability

The pollen fertility of *CaHKT1*-OE and WT plants was evaluated using Alexander staining (Alexander 1967). Unopened flower buds were collected 1 day before anthesis between 8 and 8:30 AM and crushed using an Alexander stain. Slides containing crushed samples were visualised under a light microscope (Eclipse 80i; Nikon). The fertile pollen grains inside the anthers were dyed red, whereas the sterile pollen grains remained green.

4.18 | Scanning Electron Microscopy

Anthers and ovules from *CaHKT1*-OE and WT plants under salinity stress were fixed in FAA solution (3.7% formaldehyde, 50% ethanol, and 5% acetic acid), followed by an overnight treatment with 0.2% osmium tetroxide and dehydration using an ethanol series (Narnoliya et al. 2019). The samples were then subjected to critical point drying and imaged using a scanning electron microscope (SEM; Evo LS25; Zeiss, Oberkochen, Germany).

4.19 | Complementation Analysis in *Arabidopsis*

To perform a genetic complementation assay on *Arabidopsis athkt1* mutants (ABRC stock: CS6531), a fusion construct, ProAtHKT1: CaHKT1, was generated by overlapping PCR using the *CaHKT1* CDS from ICC 9942 and the AtHKT1 promoter from Col-0. The construct was cloned into pCambia1302 via restriction-based cloning and transformed into *Agrobacterium tumefaciens* strain GV3101. Transformation of *athkt1* plants was performed using the floral dip method (Clough and Bent 1998). T_1 seeds were selected using hygromycin, and resistant plants were advanced to the T_3 homozygous generation for salinity stress phenotyping.

4.20 | Histochemical Staining for Reactive Oxygen Species (ROS)

ROS detection under salinity stress was performed using NBT staining, as described by Jambunathan (2010). Leaves from WT, *athkt1*, and complemented *Arabidopsis* lines were immersed in 0.2% NBT solution and vacuum-infiltrated at 100–150 mbar for 1 min, which was repeated 5–6 times. Samples were incubated for 15 min and exposed to fluorescent light for 20 min to produce

blue precipitates. Chlorophyll was cleared with 96% ethanol at 40°C, and leaves were fixed in ethanol:glycerol (3:1). Images were captured at 5× magnification using a Nikon 80i microscope (Nikon).

Author Contributions

J.K.M. performed experiments, performed data analysis, and drafted the manuscript. A.Y. assisted in the dual luciferase experiment and helped in drafting. L.N. and V.T. helped in scanning electron microscopy. S.T. helped with field phenotypic evaluation. S.S. and D.R. helped with the hairy root experiment. S.S., P.A., and S.K.P. conceived the idea and guided, participated in drafting, and correcting the manuscript critically, and all authors gave the final approval of the version to be published.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in the Supporting Information (Figure S1–S4; Table S1–S4) of this article. All sequencing data generated in this study is submitted in NCBI-Sequence Read Archive database (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA271716>) with BioProject ID: PRJNA271716; (Accession detail: Table S1). All high-quality genotyping information of SNPs is provided in the Tables S4 and S5 for unrestricted public access.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** A broad phenotypic variation of seed yield trait in a diversity panel of 291 chickpea accessions and 216 RIL mapping individuals used for GWAS and QTL mapping, respectively. **Figure S2:** Structural and functional annotation of *CaHKT1*. **Figure S3:** The fold expression changes of HKT1 in mutant *athkt1* and complemented lines. **Figure S4:** Cross-sectional view of roots showing tissue-specific sodium (Na⁺) accumulation in WT and *CaHKT*-OE lines, visualised using the sodium-specific fluorescent dye CoroNa Green. **Table S1:** List of 291 chickpea germplasm accessions used to constitute a diversity panel to screen the salinity stress response in chickpea. **Table S2:** Primers used for real-time PCR assay, gene cloning, and generating fusion constructs by overlapping PCR in *Cicer arietinum* (*Ca*) and *Arabidopsis thaliana* (*At*). **Table S3:** Descriptive statistics of seed yield traits measured in the diversity panel and RIL population of chickpea