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REVIEW ARTICLE

Improving Salt Tolerance of Chickpea Using Modern Genomics Tools and Molecular Breeding

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Abstract: ***Introduction*:** The high protein value, essential minerals, dietary fibre and notable ability to fix atmospheric nitrogen make chickpea a highly remunerative crop, particularly in low-input food production systems. Of the variety of constraints challenging chickpea productivity worldwide, salinity remains of prime concern owing to the intrinsic sensitivity of the crop. In view of the projected expansion of chickpea into arable and salt-stressed land by 2050, increasing attention is being placed on improving the salt tolerance of this crop. Considerable effort is currently underway to address salinity stress and substantial breeding progress is being made despite the seemingly highly-complex and environment-dependent nature of the tolerance trait.

***Conclusion*:** This review aims to provide a holistic view of recent advances in breeding chickpea for salt tolerance. Initially, we focus on the identification of novel genetic resources for salt tolerance *via* extensive germplasm screening. We then expand on the use of genome-wide and cost-effective techniques to gain new insights into the genetic control of salt tolerance, including the responsive genes/QTL(s), gene(s) networks/cross talk and intricate signalling cascades.

Keywords: Chickpea, DNA markers, Genomics, Molecular breeding, RNA-Seq, Salinity, Tolerance, Transcript, QTL.

1. INTRODUCTION

1.1. Chickpea as a Global Food Staple

Chickpea (*Cicer arietinum* L.) is the second most important grain legume and earliest known cultivated species [1]. Among grain legume crops, chickpea is highly valued for its dietary proteins, low fat, vitamins and essential minerals [2, 3]. Chickpea accessions exhibit large variance in genetic and phenotypic characteristics across the Mediterranean region, western Asia, central Asia and India, and recent breeding advances has led to its adoption to temperate regions [1, 4, 5]. The leading chickpea producing countries include India, Australia, Pakistan, Myanmar and Ethiopia, which collectively contribute more than 85 per cent of the global production. Over the past 50 years, annual chickpea production has increased from 6.4 to 14.2 million tonnes [6]. From years 2009 to 2013, the global area under chickpea increased 25 per cent from 11.5 to 13.5 million hectares [6]. The long tap root system and ability to establish symbiotic relationships with *Rhizobia* enables chickpea to fix atmospheric nitrogen and improve soil health. Given its high nutritional content, market value, adaptability and nitrogen fixation ability, chickpea is being increasingly recognised as a staple food crop of the future.

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1.1.1. The Increasing Importance of Salinity Tolerance in Chickpea

Chickpea has an indeterminate growth habit, *i.e.* it continues to grow vegetatively even after flower initiation [7]. This renders it sensitive to a number of environmental factors such as salinity, drought, heat and cold [8, 9]. Soil salinity is a major constraint that limits crop productivity and almost 80 million ha of the worlds' arable land is prone to this stress [10]. Globally, 20 per cent (45 million ha) of irrigated and 2 per cent (32 million ha) of dry land are constrained by salinity [11]. This is predicted to expand to 50 per cent worldwide by the second half of the 21st century [12]. In conjunction with the predicted marked expansion of salinity-affected area, an additional two billion people are anticipated to inhabit the planet by 2050. Therefore, soil salinity is a major stumbling block to meeting the predicted global food demand by 2050. Abiotic stresses account for about 6.4 million tonnes in crop yield losses every year, where soil salinity is a major environmental stress [13]. The enormity of the current challenge of sustaining or increasing productivity to meet yield demands in the face of increasing salinity has been well highlighted [11, 14-16]. This translates into an urgent requirement for improved crop production by almost 70 per cent [6, 17]. However, salinity limits the plant growth and severely affects the reproductive processes, resulting in lowered crop yields [18] and chickpea is intrinsically salt sensitive unlike cereals [10]. The imperative is

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therefore to elucidate the genetic architecture of salinity tolerance and in particular the molecular mechanisms underpinning the tolerance responses.

The phenotypic responses to a stress are due to a plant's genetic constitution and genotype interaction with the environmental variables [19, 20]. Existence of large genetic variation within a gene pool would enable genotypes conferring desirable traits to be identified, including those that are able to withstand adverse saline conditions [21, 22]. Salinity tolerance is conferred by several physiological factors which have been recorded in response to salt stress to identify tolerant/sensitive genotypes [23, 24]. To enable more strategic and precise selection of tolerant genotypes, frontier genomics technologies, such as gene expression profiling, have the potential to identify robust transcripts/candidate gene(s) and their alleles that condition tolerance [25-28]. Recent advances have substantially illuminated the mechanisms of salinity tolerance in chickpea, thereby paving the way towards the strategic incorporation of tolerance-imparting component traits into elite genetic backgrounds. Here, we offer a critical overview of the different genomics approaches that have been used to address salinity in chickpea. Later follows a brief discussion on targeted breeding aided by genomics tools, concluding with the future research needs for developing salinity tolerant chickpea.

2. QUANTIFYING GENETIC VARIATION FOR SALINITY STRESS TOLERANCE

Chickpea is a salt sensitive crop [10], and broad germplasm has been assessed for tolerance and subsequent deployment as parental genotypes in breeding programs [3, 21, 24, 29, 30]. Salinity tolerance has been studied at different developmental stages such as germination [31], vegetative growth [29, 32] and reproduction. Large genotypic variation was observed among chickpea landraces [29], and core collection [33], based on shoot biomass in soil treated with NaCl at vegetative and maturity stage in glasshouse experiments [21, 24]. The chickpea plant was reported to suffer most severely under salinity stress during the reproductive stage [7, 18, 19, 21, 34]. A greater number of flowers are considered to be a more important measure of tolerance during stress rather than acquiring greater root or shoot biomass [24, 35]. For example, Vadez [18], found that tolerant lines produced 70 per cent and 30 per cent more flowers under salt stress at sowing and flowering, respectively [35, 36]. Physiological studies have shown that salinity delays flowering time and severely affected during the pod filling stage. Despite pollen viability, sensitive genotypes show higher occurrence of empty pods and seed abortions [24, 36]. This observation suggests failure in ovule fertilisation as the main reason for pod abortion or empty pods, despite the viable pollen and pollen tube growth.

Further, differences in tissue ion-regulation in root, shoot and floral parts are reported among genotypes in studies that investigated the tissue/stage specific effect of salinity by measuring the $Na^+/Cl^-/K^+$ ion concentrations [7, 18, 20, 24, 35, 37]. Therefore chickpea has been shown to exhibit variable responses to high ion levels that in turn condition their ability to be tolerant or sensitive when the ionic concentration reaches a physiological threshold.

Plants employ different mechanisms such as ion-exclusion and tissue tolerance to overcome ionic stress [11, 37-39]. Reducing the (Na^+, K^+ and Cl^-) ion accumulation in shoots by manipulating the root ion transport processes was used to explain the ion-exclusion mechanism in plants [11, 40]. However, this may not be the case with chickpea where tolerant and sensitive genotypes have equal ionic concentrations in shoots [36, 37]. Also, fully expanded leaves maintained high Na^+ and Cl^- ion concentrations compared to reproductive organs during the pod filling stage and eventually restricted ions from accumulating in flowers and developing ovules [11, 41]. This suggests that the salinity effect in chickpea is minimised by compartmentalisation of toxic ions in leaf vacuoles, a process that has not yet been studied [42-45]. There is a need to investigate these cellular events in detail, to help unravel the salt tolerance mechanism in chickpea [37]. Indeed, during the pod filling stage, there is no accumulation of Na^+, Cl^- ions in reproductive organs such as petals, stamens or ovules in contrast to that detected in fully expanded leaves [24, 36]. It is possible that high ion-accumulation in leaves resulted in decreased photosynthesis efficiency and therefore tolerant genotypes (such as *cv*. Genesis836, JG11), differ from sensitive genotypes (such as *cv*. Rupali) in terms of chlorophyll content when subjected to saline conditions [46, 47]. Importantly, as mentioned shoot ion accumulation does not translate into reduced shoot biomass and seed yield [18], therefore tissue ion regulation in leaves during the reproductive stage is likely responsible for imparting a major part of the tolerance. Apart from identifying genotypic variation in already existing germplasm, there is a need to identify the molecular mechanisms pertaining to ion regulation.

3. APPROACHES TO ELUCIDATE SALINITY-ASSOCIATED CANDIDATE GENES/DNA MARKERS IN CHICKPEA FOR BREEDING APPLICATIONS

3.1. Functional Genomics

Cultivated chickpea has a narrow genetic base [1], and possesses phenotypic plasticity [48], which makes it difficult to identify the salt stress responsive and potentially tolerance gene(s) especially when plants adopt cross-talk to respond to various simultaneous stresses [49]. Therefore, there it is important to identify the transcript variants specific to salt tolerance by analysing variation in the spatio-temporal gene expression of tolerant/sensitive genotypes within a controlled environment setting. Accordingly, a large set of 20,162 Expressed Sequence Tags (ESTs) was identified from NaCl-treated roots of salt tolerant (JG11) and sensitive (ICCV2) genotypes using Sanger sequencing [50]. The functional annotation of the ESTs was achieved by similarity searches against model legume datasets (*Medicago, Glycine, Lotus,* and *Arachis*) and model plants (*Oryza*, *Arabidopsis*). Differentially expressed genes were reported to be associated with "cellular processes", "cell transport" and "osmotic adjustments". Interestingly, cDNA libraries of the sensitive genotype ICCV2 presented a higher number of up-regulated gene(s) having putative functions like heat shock proteins, metallothionein and abscisic acid production [3, 28, 50]. This suggested that chickpea plants adopt an ion-transport mechanism for regulating cellular homoeostasis through trans-membrane protein conformational changes [51], and

detoxify the metal ions accumulating beyond a threshold level. However, ESTs only represent low transcript abundances [52], and do not provide tissue or developmental stage differential gene expression within the genotypes, which are thought important to understand salinity tolerance. Serial analysis of gene(s) expression (SAGE) represents another approach to quantify transcripts exhibiting differential responses to salt stress in root and nodule tissues. Super-SAGE and DeepSAGE were used in chickpea to identify over 3,000 stress-responsive transcripts, where several *SOS* gene candidates were expressed spatially in roots [38, 53]. Subsequent microarray-based gene expression profiling of chickpea genotypes revealed a set of differentially expressed genes [54], at different time-points and in different tissue-types [38]. Earlier, Mantri *et al.* [55], reported the tolerant genotype (CPI 060546) had a higher number of repressed gene(s) than the sensitive one (CPI 60527) at different time-points (Table **1**), potentially indicating the slowing down or turning off of other non-essential metabolic processes to re-direct resources towards the tolerance mechanisms.

Importantly, genotypes differ in temporal gene regulation and a greater number of genes were down-regulated in the tolerant genotype in all of the tissue-types analysed in response to salt stress. This provided great insight into the differential transcriptional response programming among tissue types that is activated on perceiving the salt stress [28, 54]. For example, trans-membrane channels such as aquaporin genes, which transport water, were repressed much earlier in tolerant roots (24 hpt) than sensitive roots (48 hpt) to restrict the salt uptake along with water on exposure to salt treatment [55]. Importantly, both physiological and genomic screening demonstrated that a large genetic variation for salinity tolerance exists among and within the tolerant and sensitive genotypes. As an example, in microarray gene profiling, aquaporin genes were induced in tolerant-1 and repressed in tolerant-2, simultaneously [36, 55]. Other candidates such as heat shock proteins, proline-osmolytes, senescence-associated genes and ripening-related genes were repressed in tolerant roots/shoots, while the same genes were induced in sensitive roots/shoots. The identification of tissue- specific differentially expressed transcripts suggests major transcriptional reprogramming at the cellular level, which in turn confers the genetic variation by altering the plant's physiological responses to other processes such as photosynthesis [37] and senescence [24], to impart tolerance. Although these techniques provided remarkable information to identify the candidates for salt tolerance, they were restricted by the lack of a chickpea reference genome at the time of their application [63] and hence were limited to assessing known transcripts such as those represented on microarrays. They were also relatively low-throughput and failed to detect low expressed or rare transcripts, splicing events and gene isoforms which are thought to be master switches in regulating stress responses.

In recent years, advances in Next Generation Sequencing (NGS) have enabled easy access to identify thousands of gene(s) that are regulated in response to abiotic stresses in plants [28, 64-66]. A global view of the salt-stressed transcriptome using RNA-Seq would enable the measuring of gene expression responses at the whole genome level [26, 28], and provide an in-depth understanding of molecular mechanisms and pathways conferring salt tolerance. In this approach, total RNA from control and stressed root tissues are fragmented and cDNAs are sequenced with enough depth (~40 million reads per sample) to generate short (~100 bp - 150 bp length) sequence reads. The generated reads are then mapped to the now publically available chickpea reference genome (http://dx.doi.org/10.5524/100076) to obtain the "gene count values" in order to measure the differential gene expressions. Along the same lines of technology, RNA-Seq is an innovative approach which allows identification of novel transcribed loci, exon and intron boundaries and splice isoforms through reference-guided assembly. Unlike ESTs and microarrays, RNA-Seq presents a robust in-depth list of candidate genes along with rare and low expressed transcripts that facilitates understanding of the tissue/stage-specific transcriptional reprogramming in response to salt stress [52]. In a recent RNA-Seq study, an additional 15 per cent of novel transcribed loci were identified than previously annotated in the chickpea (CDC Frontier) reference genome [28]. Until now, important biological events such as alternative splicing which control the transcriptional regulation of gene isoforms during the stress were not researched [67, 68]. A transcribed locus on an average can encode for more than two exons, *i.e.* one gene can encode more than one protein and several biological processes like water transport, protein modification and defence response are reported to be alternatively spliced at different developmental stages during salt stress [28, 69, 70].

Deep-sequencing technologies generate large genic datasets to unravel the transcriptome response and understand its diversity based on developmental stage, tissue type, genotype and salt treatment [28, 66]. Several studies have demonstrated the use of RNA-Seq to study the transcriptome response of a specific cell-type such as radial patterning of root growth, anther maturity and pollen tube growth during abiotic stress responses in plants [71]. Large set of genes (5,523) were reported to be differentially expressed in chickpea in response to salinity, mostly at the late reproductive stage in root tissues [28]. This is in accordance with phenological studies where salinity is most disastrous at reproductive stages and therefore genes differentially expressed amongst sensitive and tolerant genotypes at this particular stage provide an important basis to uncover the underlying molecular mechanisms [72].

It is important to draw useful biological meaning from huge dataset generated through RNA-Seq and therefore co-expression of these genes are further analysed using computational methods to assign Gene Ontology (GO) terms to categories such as cell wall biogenesis, oxidative stress, protein folding, redox-signalling and transport [28, 63]. A set of genes enriched in particular ontology categories will enable the identification of the master regulators of complex pathways regulating salt stress responses [73, 74]. Further, given the sensitivity of the RNA-Seq approach, it is now trivial to identify the exon-junctions and intergenic non-coding regions to further study the role of small RNA molecules such as miRNA, siRNA and lincRNA, which are thought to manipulate gene functions during stress responses [75, 76]. This gene information can be used to understand the dynamics of gene networks in response to salt stress. Also, these genes can be used for developing DNA marker resources [25, 50]

Table 1. List of recently developed functional genomic resources in chickpea relevant to major abiotic stresses.

Stress	Tissue	Method	Gene/ESTs/transcripts	References
Salinity	Root	RNA-Sequencing	5545	[28]
	Seedlings	Subtractive cDNA libraries/ Yeast One-hybrid assay / Northern and western blot analyses	CaZF gene (C2H2-zinc finger family protein)	[56]
	Seedlings	Semi-quantitative RT-PCR	CapLEA-1 CapLEA-2, CarLEA-4 (late embryogenesis abundant protein)	[57]
	Hooks, epicotyls, mesocotyls from seedlings. Stems Leaves, pods, flowers and roots	Northern/Southern blot analyses	CapLTP (lipid transfer proteins), CapLEA-1 CapLEA-2, CarLEA-4 (late embryogenesis abundant protein)	[58]
	Seedlings	cDNA	SOD (cytosolic superoxide dismutase)	[58]
	Seedlings	RNA-Blot Hybridization and RT-PCR/ Site-directed mutagenesis/ Yeast One-hybrid assay	CAP2 gene (an APETALA2-family transcription factor)	[59]
	Root, shoot, leaves, stem, flowers, young pod and seedlings	RNA-Sequencing	1163 genes	[26]
	Root and nodules	deepsuperSAGE	21401 transcripts	[38]
Salinity and Drought	Leaves, apical meristem, shoots, roots, buds, flowers, pods, embryo	Tentative unique sequences (TUSs) using Roche454 and Sanger ESTs/ Illumina/Solexa sequencing	103 215 ESTs	[25]
	Leaves, roots, flowers	'Pulse Chip' microarray/RT-PCR	266 (salt responsive transcripts)	[54]
	Root	ESTs	20162 cDNAs	[50]
		SuperSAGE	3000 transcripts	[60]
	Seedlings	subcellular localization/qRT-PCR	CarF-box1(CarF-box1 protein)	[61]
Salinity, Drought, Cold	Leaves, roots, flowers from tolerant	'Pulse Chip' microarray/RT-PCR	386 (salt responsive transcripts)	[55]
Salinity, Heat, Environmental stress	seedlings	Quantitative real-time PCR/Northern Analysis	CaMIPS1, CaMIPS2 (L- myo-inositol 1-phosphate synthase)	[62]

or act as candidates for genomics-assisted breeding and prediction of phenotype from genotype [77]. The RNA-Sequencing technique is continuously improving to overcome issues like sequence coverage or 3' fragmentation bias through longer read length, paired-end sequencing, cation-heat fragmentation, stranded library and random hexamer priming during cDNA synthesis [52, 66].

3.2. Mapping of Genomic Regions Relevant to Salinity Tolerance

Salinity tolerance is a physiological and biochemical trait controlled by many genes/quantitative trait loci (QTLs) exerting variable contributions to the tolerance phenomenon [3, 18, 19]. Limitations of conventional genetic analyses in precisely delineating causative gene(s)/genomic segments conditioning tolerance to salinity are largely due to the complex interactive mechanisms at play. The lack of selectable phenotypic variation for salinity tolerance in the cultigen has greatly impeded successful breeding of tolerant cultivars [78, 79]. Alternatively, selection of the genetic components underpinning the tolerance traits would potentially speed up the accuracy and timing of tolerance breeding. Several methods including QTL discovery, using family- or population- based mapping, were applied to identify the genomic position of the genetic determinants governing the various aspects of the salinity tolerance trait. For example, Samineni *et al.* [80] identified a set of QTLs/genes related to yield, seed size and

shoot biomass under salt-stress using a RIL population (ICC6263 × ICC1431). Although 20 candidate genomic segments were implicated, these accounted for just 9 per cent of the total trait variation, highlighting the complex and multigenic nature of the salinity tolerance trait. Similarly, Vadez *et al.* [18], identified multiple QTLs for salinity tolerance using another RIL population (JG62 × ICCV2) on linkage groups (LGs) 3 and 6, including a major QTL on LG 6 that accounted for 37 per cent of the variation in seed number.

Most recently, genomic regions associated with salinity tolerance were identified on LGs 5 and 7 using the RIL population (ICCV2 × JG11) and these segments corresponded to 48 putative candidate tolerance genes [3]. These QTL regions explained higher reproductive success under salinity and can be potentially used in marker-assisted breeding. By using the chickpea reference genome sequence information, the putative full-length candidate gene(s) underlying these QTL regions could be identified and annotated with respect to their structural and functional features. The syntenic regions of tightly linked markers to QTL were BLAST against whole genome sequence to find candidate genes [3], which have putative role in phytohormone signaling pathways and Na+/K+ antiporter ion channels such as AKT1 [3, 81, 82].

Salinity tolerance is also influenced by epistatic and environmental (G×E) interactions offering an additional set of challenges to breeding efforts [20, 33, 83]. Future functional validation of these candidate sequences will determine the magnitude of their functional relevance to salinity tolerance across a broad set of genotypes and treatment environments, thus substantiating their suitability as selection tools within breeding programs.

3.3. Molecular Mechanisms Underpinning Salinity Tolerance in Chickpea

Plants have developed different sensory mechanisms to cope up with salt stress. The signalling mechanism is regulated first at the plant hormonal level [84], second by activation of transcription factors for gene expression and third by activation of metabolic pathways (Fig. **1**).

The plasma membrane is the first line of defence which perceives the stress through trans-membrane protein sensors [39]. The phospholipids like Phosphatidylcholine (PC) and Phosphatic Acid (PA) receive the signal when high extracellular NaCl concentration occurs at ionic receptors of the root cell [85]. These phospholipids have specific roles in regulating activation of calcium-dependent protein kinase *Ca*CDPK1 genes, which are involved in the release of signalling messengers such as calcium ions (Ca^{2+}) [86, 87] through the control of transcription factors during the saline stress responses in chickpea [88]. Transcription factors bind to the promoter regions of the genes to facilitate the RNA polymerase to start the transcription and subsequent translation of the gene products [89]. They are important regulators of stress response and have been widely found to show differential expressions in salt-challenged tissues [25, 26, 90]. Crops like *Arabidopsis, Oryza* and *Glycine* present a robust suite of functionally annotated candidate gene(s) and have been extensively studied to understand molecular mechanisms involved in abiotic response through gene manipulations. A number of transcription factors in these crops have been identified to be associated in the activation of genes responsible for osmotic adjustments [59, 91, 92]. Transcription factors such as *CAP2/AP2*, *CarNAC1*, *CaZF* and *CarF* that are known to up-regulate the *Ca*CDPK1 genes have been identified in chickpea [26, 61].

Recently, the chickpea F-box gene *CarF-box1*, isolated from a cDNA library of polyethylene glycol (PEG)-treated chickpea seedling leaves, was significantly induced in roots following drought and salinity stresses [61]. Several transcription factors such as *CAP2*/*AP2*, *CarNAC1*, *CaZF* and *CarF* were identified from stressed tissues through generation of cDNA [61, 93] and genomic libraries [26]. These TFs were reported to regulate gene expression mediating hormonal biosynthesis and subsequent plant growth under stressed conditions [94, 95]. Overexpression of the *CAP2* gene resulted in an increased tolerance to dehydration and salt stress [59, 93]. Another chickpea abiotic stress transcription factor *NAC* gene, *CarNAC1* and *CaZF*, which imparted high salinity tolerance when expressed in tobacco plants, was isolated from a cDNA library constructed with PEG-treated seedlings [56, 91, 96]. Concordant with the earlier reports, chickpea is known to accumulate increased inositol during dehydration stress [97, 98], and these TFs regulate expression of *MIPS* (Myo-inositol-1-phosphate synthase) genes, aiding the cell to maintain the osmotic environment [62]. Previously, *CaMIPS2* was reported to be present as an ABA-inducible early dehydration-responsive gene in chickpea and therefore, an important component of hormonal signalling [49].

Further, during the response to salinity stress in chickpea, Lipid Transfer Proteins (LTPs) form an impermeable layer, which obstructs water loss and retains cell turgidity [57]. The *CapLTP* gene in chickpea was expressed in young tissues and during early developmental stages in response to water stress, suggesting implications for protecting cellular functions from damage caused by high ion concentration.

The activation of TFs and gene transcription is highly dependent on up-regulated plant hormones such as ABA, Indole Acetic Acid (IAA), gibberellic acid (GA) and methyl jasmonate (MeJA) [49, 99], which induce expression of *CarLEA* genes. Accordingly, *CarLEA* genes (*CarLEA1*, *CarLEA2* and *CarLEA4*) isolated from chickpea cDNA libraries, were found to impart desiccation tolerance during seed development, thereby protecting plants against a variety of stresses, including drought, salinity and freezing [57, 58].

Plant hormones such as ABA, SA, JA induce signalling cascades and protect cells from osmotic imbalance and dehydration. Several ABA-responsive transcripts were identified in relation to various abiotic stresses in chickpea [88], however the role of this hormone in the signalling for salt stress tolerance remains unclear. Likewise, Salicylic Acid (SA) is recognized as an endogenous signalling molecule and major elicitor of ROS, Phosphatidic Acid (PA) and cellular proteins like annexin [84]. SA evokes environmental stress responses by regulating nutrient uptake, photosynthesis, osmotic balance and seed germination. Several ion-channel and membrane transporters are located in the plasma membrane, and elucidation of this pathway triggered by PA production as a result of SA accumulation could be very important in the salt stress response.

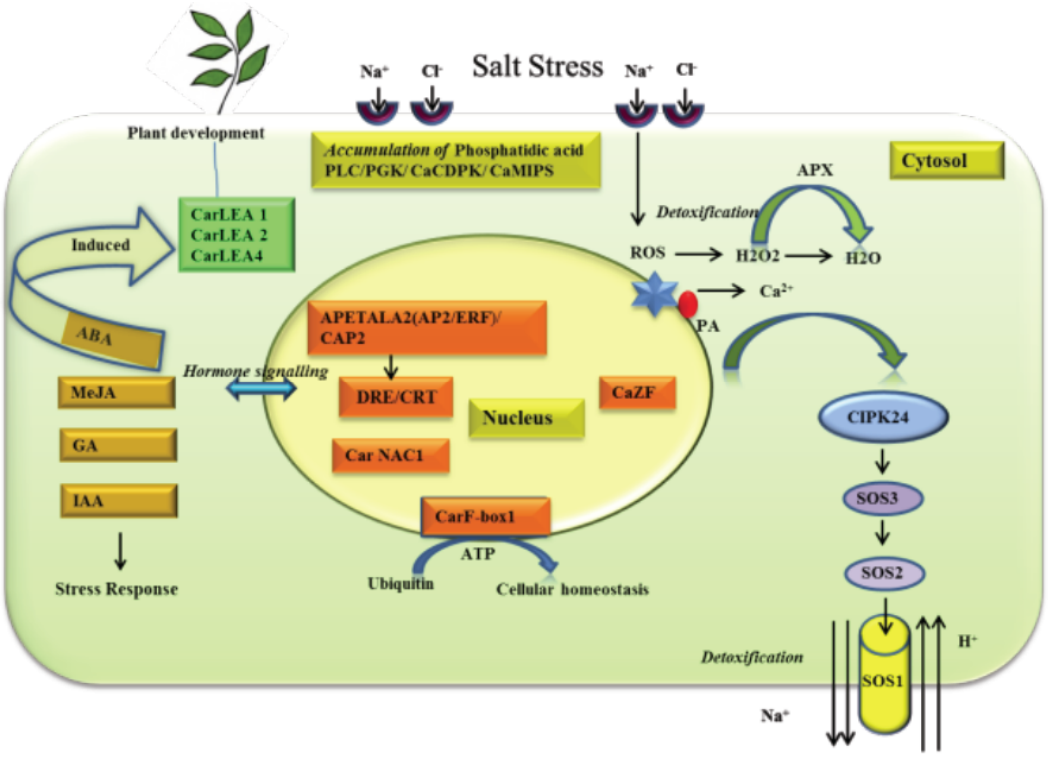

Fig. (1). Overview of the proposed salinity tolerance mechanism in *Cicer arietinum* L. Upon salt stress, Ca^{2+}, ROS and hormone signalling are activated. *AP2*/*ERF*, *CAP2*, *CarNAC*, *CarF* box-1 type transcription factors have been reported to overexpress at the stress reception. The Salt Overly Sensitive (SOS) pathway regulates the Na^+/ H^+ antiporters. Ubiquitin, Ionositol, ABA, MeJA and salicylic acid pathways are induced by gene(s) such as *CarLEA* (*Cicer arietinum* late embryogenesis protein).

As indicated in the preceding sections, there is substantial evidence that ions accumulate in different tissues and cell organelles under salinity stress [45, 100, 101]. Plants deploy various pathways to regulate the ionic detoxification process by importing proton (H^+) and exporting Na^+ out of the cell [102]. One major ion-exchange protein (*NHX*) has been located within the plasma membrane and proposed to control the detoxification process by the efflux of excess Na^+ ions and influx of H^+ ion in *Arabidopsis* [103-105]. However, not enough is known about the molecular mechanism and pathways that are involved in these detoxification processes, the sequestration of ions into vacuoles through compartmentalisation or ion-exclusion in chickpea.

To date, few studies have uncovered candidate genes in chickpea such as *SOS1*/Na^+/H^+ antiporter, *SOS2*/*CIPK24*, *SOS3*/*CBL*, associated with the SOS pathway. These have a putative role in excretion of Na^+ ion suggesting ion-exclusion mechanisms occur under high salinity concentrations in chickpea [38]. However, more comprehensive studies are required to identify the members of SOS signalling pathways that invoke the salt stress response in chickpea. There is a great need to employ technologies like RNA sequencing to elucidate in more depth the signalling cascades and gene-networks that are crucial for salt tolerance.

4. POTENTIAL STRATEGIES TO BREED SALT STRESS TOLERANT CHICKPEA

An in-depth understanding about the genetic determinants of salt tolerance is an essential prerequisite for selective biotechnological manipulation or molecular breeding towards developing salt tolerant cultivars. In important crops like barley, strategies have focused on targeted modification of osmotic tolerance and ion exclusion mechanisms, and these have resulted in high tolerance to tissue ion concentrations [104, 106]. Given that high tissue ion tolerance was recently identified as a key mechanism for salt tolerance in chickpea, it is logical to propose that a strategy to improve the ion channel regulation mechanisms would be most relevant for improving salt tolerance in this species.

Molecular breeding methods are now available that facilitate effective translation of the genomic knowledge for the development of tolerant varieties. Screening the available germplasm to identify tolerant accessions is the foremost approach. As previously discussed, chickpea germplasm collections were examined by many researchers with the aim to discover salt-tolerant genotypes [21]. This has resulted in the identification of a set of salt tolerant genotypes, which could be used as a genetic reservoir to mine salt responsive and potentially tolerance-related gene(s)/QTLs, and also, to facilitate transfer of the corresponding alleles into elite yet vulnerable elite genetic backgrounds [13]. A set of specific physiological indicators may be selected while assessing salt tolerance among chickpea genotypes. With the advent of next generation phenomics platforms such as robotic field sensors high resolution multi-spectral mapping using UAVs and laser light back scattering technology, it has become easier to study developmental stages of plant more precisely and analyse the multi-dimensional large volume of bio-imaging data [107-109]. The important parameters involve

germination, biomass, leaf necrosis, nodulation and nitrogen fixation, death and senescence, ion concentrations, osmoregulation, plant growth and yield. Each has been used previously with varying effectiveness for the selection of salinity tolerant plants [36]. The factors that challenge accurate evaluation of genotypic tolerance include: 1) the complex genetic control of salinity tolerance 2) time consuming screening protocols and 3) substantial G × E effects.

Similar to other cultivated legume species, chickpea has a narrow genetic base, which has been severely impacted through domestication and subsequent selection events [1]. Alternatively, mutation breeding is an attractive approach to broaden the available genetic diversity, and to create novel variability. This approach has already been employed to develop several chickpea varieties [110], and the modern variants of mutation-detection systems like TILLING-by-sequencing [111], create novel opportunities to tap variations related to abiotic stress tolerances. Other potential approaches enabling "siphoning" of exotic/wild alleles include the use of crossable wild relatives. Wild chickpeas do possess tolerance to a number of abiotic stresses. However, potential of these wild relatives has to be realized concerning salt tolerance using conventional breeding protocols. Toward this end, recent genomic techniques like advanced backcross (AB)-QTL and introgression libraries may be particularly relevant for capturing the beneficial yet previously unnoticed exotic alleles [112].

Advancements in the field of crop genomics, with copious sequence data now available, help scientists to identify, isolate and deploy the genes associated with the tolerance traits [77, 113]. Once candidate sequences have been identified and functionally validated, salt tolerant chickpea cultivars may be routinely developed using modern breeding techniques such as Marker-Assisted Selection (MAS), marker-assisted backcrossing (MABC) and marker-assisted recurrent selection (MARS) [114]. Also, with the availability of high-density marker genotyping assays and high-throughput phenotyping platforms, opportunities are created to employ Genome Wide Association Studies (GWAS) and Genomic Selection (GS) to counter the issue of QTL with relatively smaller effects for salt tolerance [115, 116]. The latest applications of NGS technology have rendered identification and mapping of DNA markers a rapid and cost-effective procedure [117]. The high density genotyping is a common occurrence now with the availability of diverse protocols such as Reduced Representation Libraries, Restriction site Associated DNA sequencing (RAD-seq), Genotyping by sequencing (GBS), low-coverage Whole Genome Resequencing (WGRS) or genome skimming [118], and a more recent, Single Locus Amplified Fragment Sequencing (SLAF-Seq) [119]. Also, development of genome-scale catalogues of genetic variants such as SNP chips including SoySNP50K iSelect BeadChip [120], SoySNP6K Infinium BeadChip [121], Axiom SoyaSNP array for nearly 180,000 SNPs greatly assists genetic analyses [122]. The genome-wide SNPs in combination with high-quality phenotyping records permit genetic resolution of trait mapping at an unprecedented scale. For instance, WGRS recombination bin map based QTL analysis in chickpea allowed splitting of the single 3-Mb QTL hotspot region into two precisely delineated genomic regions (139.22 kb and 153.36 kb). Concerning salinity tolerance, a refined trait dissection facilitated by high-density SNP data is evident in a recent GBS assay of RILs, which elucidated salinity tolerance in rice to be controlled not only by additive but also epistatic interactions [123]. The average QTL interval size was 132kb. In another study using BSA with 50K SNP array, authors discovered known as well as novel QTLs for salinity tolerance in rice, with an average QTL region of 2.3 cM [124]. In soybean, the WGRS of RI panel (W05 × C08) at 1x revealed a 978-kb QTL region for salt tolerance, which was further narrowed down to 388-kb. The corresponding 388-kb of W05 with William 82 led authors to propose a major dominant gene Glyma03g32900 (GmCHX1) as the causal one [125]. This locus was also later detected by Patil *et al.* [126] through conducting GWAS on publically available WGRS data of 106 diverse soybean lines, leading to design of KASPar assay to support breeding for salt tolerance. In view of the availability of the reference and re-sequenced genomes in chickpea, such approaches could be extended to comprehend the genetic makeup of salinity tolerance in chickpea followed by fine-mapping, prioritization of the candidate genes and pyramiding of candidate genes.

In view of the deluge of genome-scale sequencing data as described above, now is an opportune time to characterise the ion-transport channel genes such as Hydrogen/potassium Exchanger (HKT), Cation/proton Exchanger (CHX), sodium/hydrogen exchanger (NHX), as has been done in major crops like wheat [127], and soybean [81]. Experimental evidences collected so far support the crucial role of these genes in conferring salinity tolerance and improved yield [128]. In parallel, different metalloenzymes such as Superoxide Dismutase (SOD), Ascorbate Peroxidase (APX), peroxidase (POX) that is elicited during stress could be targeted [117-119, 129-131]. Quantifying expression levels through RNA sequencing or Digital Expression Analysis (DGA) can generate unprecedented insights into the molecular cascades of mechanisms that lead to ion-exclusion inside cells.

Developments in different next generation omics platforms have generated huge information which is useful to understand the complex genetic and physiological nature of abiotic stress tolerance. An efficient integration of genomics, proteomics, metabolomics, ionomics and phenomics will enrich our biological understanding of the salt tolerance response however it still remains a challenge in legume crops [132]. Recently, an integrated transcriptome and metabolome study in *Dendrobium officinale* provided deeper understanding of gene to metabolite network regulating energy metabolism through oxidation of carbohydrates during the cold stress [133]. Computational biology has emerged as a powerful approach to make the different omics data accessible to the community through creation of public databases. Establishment of such databases allow easy retrieval of information to examine to study the correlation of a gene to its functional protein, syntenic regions to a chromosome of model plants, end product metabolites or ionic regulation in order to predict and shape an improved phenotype for stress tolerance. Hence, there is a great need to develop databases containing information on ionomics, metabolomics and phenomics databases especially in legume crops like soybean and chickpea which have been greatly benefitted with current genomics and transcriptomics advances.

In the near future, high-resolution and annotated transcriptome/genome sequence data will lead to the development of large-scale selective breeding tools for accurate and fast salt tolerance selection. The genomic toolkit that underpins breeding for salinity-tolerant chickpea will encompass a suite of robust molecular resources that have been validated through multiple treatments and environments as well as across diverse genotypes. The selective breeding for improved production will be strengthened and help assure that chickpea remains a major and secure food source in the face of increasing salinity stress worldwide.

LIST OF ABBREVIATIONS

AGP	=	Arabinogalactan class proteins
CAP2	=	Adenylyl Cyclase-Associated Protein
CaZF	=	*Cicer arietinum* zinc finger
CDPK1	=	Calcium-dependent protein kinase
CIPK	=	Calcineurin B-like interacting protein kinases
DRE/CRT	=	Dehydration responsive element/C-repeat element
EST	=	Expressed sequence tag
LEA	=	Late-embryogenesis abundant
MeJA	=	Methyl jasmonate
MIPS1	=	Myo-inositol-1-phosphate synthase
P5CS	=	Pyrroline-5-carboxylate synthetase gene
QTL	=	Quantitative trait loci
RGA	=	Resistance gene analogues
RIL	=	Recombinant inbred line
ROS	=	Reactive oxygen species
SAGE	=	Serial Analysis of Gene Expression
SNP	=	Single nucleotide polymorphism
SOS	=	Salt overly sensitive

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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