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Research Article

Unveiling the Potential of OsRuvB DNA Helicases in Enhancing Salinity Stress Tolerance in Chickpea

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Abstract

Chickpea (*Cicer arietinum* L.) is a self-pollinated true diploid (2n=2x=16) cool season leguminous crop that ranks second among food grain legumes after soybean. It grows under a wide range of climatic conditions and is highly sensitive to salt stress. In the present study, transgenic chickpea plants (var. HC-1) carrying *OsRuvB* gene were screened for salt stress. Putative transformants were screened at an early stage through PCR amplification using gene specific primers and a transformation frequency of 36.2% was observed. Physio-biochemical analysis of selected T_2 transgenic plants subjected to 100 mM salt stress showed that transgenic plants were able to maintain higher chlorophyll content, relative water content, cell viability, proline content, Na^+/K^+ content, catalase and peroxidase activity compared to the wild type plants. Whereas electrolytic leakage and lipid peroxidation were relatively less as compared to the wild type plants under 100 mM stress. Among all transgenic lines, line 8 performed well with respect to all the parameters studied and can be taken further for the development of transgenic chickpea plants for salt stress tolerance.

Keywords: Chickpea; Transgenic; *OsRuvB;* Physio-biochemical; Salt stress

Introduction

Chickpea (*Cicer arietinum* L.) is a self-pollinated true diploid (2n=2x=16) leguminous crop with a 738 Mbp genome that ranks second among food grain legumes in the world after soybean. It is grown in a wide range of environments in over 50 countries in subtropical and temperate regions of the world [1].

Although chickpea is grown in over 50 countries, 90% of the area under chickpea cultivation is in the developing countries, with southern and South-East Asian countries accounting for >79% of the global production [2]. Globally chickpea harvested area has been expanded from 9.63 million ha in 1980 to 12.65 million ha in 2016. Global chickpea production has also increased from 4.85 million tons in 1980 to 12.09 million tons in 2016. During 2017-18, a total of 25.23 million tons pulses were produced from 29.99 million ha. Out of total pulses production, a total of 112.29 lakh tons chickpea was produced from 105.61 lakh ha area which accounted for 35. 21 per cent and 44.50 per cent of area and production of total pulses, respectively [3]. The global growth rate of pulse production over the last decade has been 2.61%. In India, Madhya Pradesh is the highest chickpea producing state with a share of 41% of the national production. The other major chickpea producing states are Rajasthan, Maharashtra, Andhra Pradesh, Telangana, Uttar Pradesh, and Karnataka, which cover 95% the area under chickpea cultivation (State wise share to total production and area of chickpea in India 2015–2016). Cultivars grown in India are either native (desi) types or Mediterranean (Kabuli) types. The growth trends of area and production of pulses in Haryana found declining from 1970-71 to 2016-17. In 2017-2018 the production of chickpea went down from 36.4 thousand tons from 32 thousand ha of area [4].

Chickpea seeds consist of 19.3% protein, 64.6% carbohydrate, and vitamins [5]. Although chickpea has a high yield potential (4000 kg/ha), actual yields are quite low due to biotic and abiotic stresses [6]. High salinity is one of the major abiotic stress factors that reduce plant growth ultimately hindering crop productivity [7]. At least 20% of all irrigated lands are salt affected, with some estimates being as high as 50% [8]. Salinity is a soil condition characterized by a high concentration of soluble salts. Soils are classified as saline when ions concentration is such that osmotic pressure produced by ions are equivalent to that generated by 40 mM NaCl (i.e., 0.2 MPa) or higher [9]. Abiotic stresses affect various morphological, physiological, and biochemical processes, though all plants in a timely and well-coordinated response such that tolerant genotypes which are well adapted adaptation and survive under stress [10]. Excess of soluble salts in the soil leads to osmotic stress, resulting in

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ion imbalances and toxicity, resulting in retarted plant growth [11,12]. Under stress conditions, Reactive Oxygen Species (ROS) are commonly generated and stored which cause oxidative damage to biomolecules such as lipids and proteins, resulting in cell death later in the process [13]. One of the approaches to overcome the consequence of salt stress is increasing salt tolerance in crop plants. Salinity is a complex trait which is associated with various cellular mechanisms [14].

Plant breeders have achieved some success in producing salt-tolerant lines/cultivars of some crops through conventional breeding; however, the main issue that conventional plant breeders have faced is the low magnitude of genetically based variation in the gene pools of most crop species. However, because of reproductive barriers, transferring salt-tolerant genes from wild relatives to domesticated crops is not easy [15] and thus very few examples of this approach being used effectively could be found in the literature [16,17]. The overall approach is time-consuming and labor-intensive; undesirable genes are frequently transferred alongside desirable ones; and reproductive barriers limit transfer of favorable alleles from inter-specific and inter-generic sources. Because of these factors, genetic engineering has emerged as an alternative strategy to conventional breeding for crop quality and impend yield potential in most crops. Nonetheless, plant biologists have focused heavily on genes that encode ion transport proteins, compatible organic solutes, antioxidants, heat-shock, and late embryogenesis abundant proteins as well as transcription factors for gene regulation to improve salt tolerance traits in various crops through genetic engineering [15]. Plants respond to stress by changing gene/transcript/protein expression levels at the molecular level. Overexpression of several stress-induced genes, including helicases, provides salinity stress tolerance in crop plants [18]. DNA helicases act as molecular proteins in a variety of cellular mechanisms and are required for nearly all DNA metabolic activities, including pre-mRNA splicing [19]. *RuvB* DNA helicases are capable of imparting salinity tolerance in *Arabidopsis*, rice, and pigeon pea [20]. *RuvB* is a member of AAA+ (ATPases Associated with diverse cellular Activities) superfamily, and part of *SF6* superfamily which belongs to helicase class. Most of the helicases belong to *DEAD*-box protein superfamily. They are involved in regulation of cellular machinery such as DNA repair recombination, replication, transcription, translation initiation, ribosome biogenesis. So, they play a crucial role in stabilization of growth during stress conditions in plants. There are reports that helicases are up-regulated in response to abiotic stress in plants and help in survival under stressed conditions. Some of the examples of helicases which are activated under abiotic stresses are *PDH45*, *PDH47, STRS1*, *STRS2, MCM6, p68* etc. [21-24]. The role of helicases has been established in various cellular functions such as replication, transcription, translation, gene regulation, DNA damage repair, chromatin remodeling and stress tolerance [25-28]. *RuvB* is a SF-6 type DNA helicase associated with diverse cellular activities such as protein folding, proteolysis, cytoskeleton regulation, and transcriptional control [29-32]. *RuvB* is well characterized in *Escherichia coli* [33], but now there are reports on characterization of *RuvB* in rice [34].

In the present study, Haryana chana 1 (HC-1) variety has been used to for the over expression of *OsRuvB.* HC-1 is a highly cultivated variety of chickpea used for commercial cultivation. India is the largest producer, consumer, and importer of pulse crops [35]. Due to the consumers and Government price support policies which are predominantly in favor of cereal crops it causes global production still lags behind [36]. Through given *RuvB* orthologous function in stress response, role in cellular functional reprogramming biotic stress we investigated its role in abiotic stress tolerance. To our knowledge, we were able to generate T2 chickpea heterologous expressing *OsRuvB*.

Materials and Methods

Materials

Plant Materials: In the present study chickpea variety, HC-1 and *OsRuvB* transgenic lines were used for studying morpho-physiological and biochemical responses of *OsRuv*B against salt stress. Seeds of chickpea were procured from Molecular Biology Biotechnology and Bioinformatics department, CCS HAU, Hisar.

OsRuvB, Plasmid and Agrobacterium tumefaciens strain: *Agrobacterium tumefaciens* strain: LBA4404 containing pCAMBIA1301 harboring *OsRuvB* gene was previously used for genetic transformation of HC-1 chickpea variety (Patent no. 252590) (Figure 1) [37]. The strain was a gift from Tuteja, N.K. ICGEB, Delhi.

Gene-specific primers: Gene-specific primers for *OsRuv*B gene used were designed using IDT software for PCR analysis. Both 557bp and 957bp primers were used for confirmation of *OsRuvB* (Table 1 & 2). These primers were synthesized from Eurofins Genomics, India Pvt. Ltd.

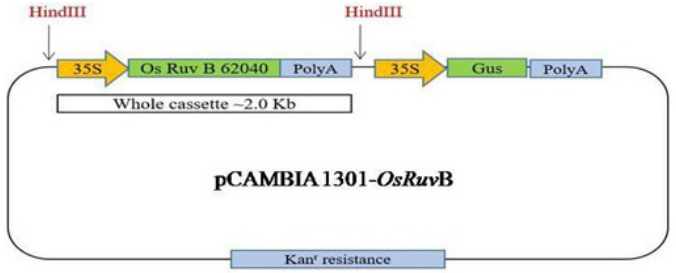

Figure 1: pCAMBIA vector harboring *OsRuvB.*

Table 1: Gene specific primer (957 bp).

Primer	Sequence (5'→3')
Forward	GTGGCAGTGGGTGATGTTAT
Reverse	ATCTCAGTGGGACGGTATGT

Table 2: Gene specific primer (557 bp).

Primer	Sequence (5'→3')
Forward	CATCTCTCAGGAGCTAGGTAGT
Reverse	GATGTCTGTTGTCCGATCTCTC

Table 3: Screening of T_2 generation plants for the presence and integration of *OsRuv*B gene.

T_1 Plants	Number of T_2 Plant Screened	Confirmed by PCR
Line 4	15	5
Line 8	18	7

Genomic DNA Isolation

DNA from the young leaves of T_2 and wild type chickpea pans were isolated using CTAB method [38]. DNA purification samples were re-extracted with equal volumes of phenol: chloroform: isoamylalcohol (25:24:1) followed by DNA precipitation and washing with 70% ethanol twice. DNA pellet was air dried, dissolved in appropriate volume of TE buffer and stored at -20°C till further use. The quality and quantity of isolated genomic DNA were estimated at 0.8% agarose gel electrophoresis.

RNase treatment: The RNA contamination in the samples was removed by adding 3 µl RNase A (10 mg/ml) to each sample and incubated at 37°C for 3-4 hours.

Purification of DNA: Samples were re-extracted with equal volumes of phenol:chloroform: isoamyl alcohol (25:24:1) fol-

Table 4: Effect of 100mM NaCl salt treatment on various physio-biochemical parameters in T_2 wild type and transgenic chickpea plants lines at 7th days after treatment.

Line	Chlorophyll Content (mg/g FW)	RWC (%)	EL (%)	Proline (µmole/g FW)	MDA (µmole/g FW)	Na^{+} (ppm)	K^{+} (ppm)	POX (µmole/g/min FW)	CAT (µmole/g/min FW)
WTNS	2.98±0.06	71.3±0.301	44.2±0.531	110±1.24	0.035±0.023	2.5±0.065	3.7±0.088	20.4±0.034	44.2±0.136
WTS	2.1±0.053	54.2±0.901	60.3±1.31	120±0.93	0.051±0.04	8.7±0.199	2.3±0.024	24.8±0.426	52.2±0.95
4LNS	3.86±0.024	79±1.692	40±0.77	150±0.156	0.028±0.07	2.1±0.04	4.7±0.051	23.56±0.76	57.2±0.774
4LS	3.68±0.073	70.3±0.256	50.2±0.82	180±1.592	0.044±0.002	6.1±0.079	2.1±0.014	33.21±0.553	60.62±0.537
8LNS	5.05±0.118	80.6±1.804	42.3±1.07	150±2.96	0.024±0.001	2.9±0.02	4.8±0.032	31.1±0.306	55.3±0.835
8LS	4.29±0.09	76.3±1.787	49.5±0.90	160±3.497	0.033±0.001	4.1±0.034	2.8±0.023	39.2±0.281	61.4±0.767

lowed by DNA precipitation and washing with 70% ethanol twice. DNA pellet was air dried, dissolved in appropriate volume of TE buffer and stored at -20°C till further use. The quality and quantity of isolated genomic DNA were estimated by 0.8% agarose gel electrophoresis.

Qualitative and quantitative estimation of genomic DNA: Quality of DNA was examined by submerged horizontal gel electrophoresis. A 0.8% (w/v) agarose gel was prepared for this (Sambrook *et al.,* 1989). Gel casting plate was washed, air-dried and its ends were sealed with tape. Agarose was melted in 1 X TBE buffer and ethidium bromide was added at a concentration of 0.5 µg/ml of the gel solution. Gel solution was then poured into casting plate inserted with an appropriate comb to get 0.4-0.6 cm thick gel. Sealing tape was removed from both the ends as the gel solidified. The quality of DNA was examined by submerged horizontal gel electrophoresis. A 0.8% (w/v) agarose gel was used [39]. Samples were prepared by adding 4 µl of sterile distilled water, 2µl of 6X dye and 1µl of DNA sample (4:2:1). Samples were loaded in the wells and electrophoresis was carried out at a constant voltage (100 V) until dye migrated to the other end of the gel. Gel was then visualized under UV transilluminator, and photo was taken using UV Gel documentation system (Benchtop UVP). Quantitative estimation of genomic DNA was done by Nanodrop.

Molecular Analysis of Transgenic Chickpea Plants Carrying *OsRuv*B Gene

Screening of putative transgenic chickpea plants (HC-1) carrying *OsRuv*B gene: The putative transgenics were screened for the presence of *OsRuv*B gene through PCR using gene-specific primers. The gene-specific primer pair used in the present study was synthesized from Eurofins Genomics, India Pvt. Ltd**.**

PCR amplification: PCR reactions were carried out in 20µl reaction mixture containing 50ng DNA, 2µl of 10XPCR buffer (G-Biosciences) with $MgCl_2$, 0.5µl of 10 mM of each forward and reverse primer (Eurofins), 0.5µl of 10mM dNTP (Thermo Scientific) and 2.5U *Taq* DNA polymerase (G-Biosciences). PCR was performed in Benchtop thermocycler.

Following PCR conditions were used: Initial denaturation 95°C for 10 min, denaturation 94°C for 1 min, annealing 52°C for 1.5 min, extension 72°C for 1.5 min and final extension 72°C for 10 min. Amplified products were stored at -20°C till further use.

Morpho-Physiological and Biochemical Analysis of Transgenic Plants under Salt Stress

Wild and transgenic plant morphology was recorded before and 7th Day after the abiotic stress treatment and photographed (Nikon D3500). Transgenic and non-transgenic chickpea plants were raised in dune sand pot and were subjected to 100 mM NaCl at flowering stage. Physiological and biochemical parameters relative water content, chlorophyll content, electrolyte leakage, lipid peroxidation, proline content, cell viability, sodium-potassium content, protein expression catalase and peroxidase activity were recorded at 7th day after salt treatment. WTNS - Wild Type under Non Stress, WTS - Wild Type under Stress, TNS - Transgenic under Non Stress, TS - Transgenic under Stress

Root morphology: The morphology of root was observed before and after stress and photograph taken.

Chlorophyll Content (mg/g FW): Leaf tissue was washed, blotted dry and dipped in test tubes containing 3 mL of Di-Methyl Sulfoxide (DMSO) overnight as described by reference method [40]. Extracted chlorophyll in DMSO was estimated by recording its absorbance at 663 and 645 nm, respectively and its amount was calculated from the formula:

Chl. a = (12.3 A_{663}–0.86 A_{645}/ax1000xW) *V

Chl. b = (19.3 A_{645}–3.6/ax1000xW) *V

Were, V: Volume of DMSO, A: Path length, W: Weight of Tissue

Relative Water Content (%): Leaf relative water content was calculated by using the method as described [41]. Leaf samples were collected and weighed immediately to measure weight. Leaves were placed separately in petri dishes filled with distilled water for 3 h. After, the same leaves (fully turgid) were weighed again and baked at 85°C for 72 h for drying and weighing and used to calculate percent Relative Water Content (RWC %).

RWC (%) = (Fresh Weight-Dry Weight/Turgid Weight-Dry weight)×100

Electrolyte leakage (%): Membrane injury was analyzed according to the standard method [42]. The electrolyte leakage was determined using a conductivity meter. One gram of fresh leaves was cut into pieces and placed in test tubes, in a water bath at 65°C for 1 hour and 20 ml of deionized water was added and kept overnight, the initial electrical conductivity of the medium, (i.e., EC1) was measured the following day.

Samples were then autoclaved for 20m for electrolyte release, cooled followed by m and final Electrical Conductivity, i.e. (EC2) was measured. Percent electrolyte leakage was calculated as follows:

Electrolyte leakage (%)=(1-EC1/EC2)×100

Lipid peroxidation (µmol/g FW): Lipid peroxidation was measured in terms of Malondialdehyde (MDA) content present in leaf tissues. MDA is a product of lipid peroxidation and was measured by Thiobarbituric Acid (TBA) [43]. Absorbance was read at 532 nm and 600 nm. The concentration of MDA was calculated using its extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Cell viability assay: Cell viability assay was determined by

Evans blue staining [44]. Evans blue release was estimated using a spectrophotometer (GENESYS 180 UV visible spectrometer) at 600 nm absorbance.

Proline content (µmol/g FW): Proline content was estimated by the reference method [45]. Proline content was calculated using a standard curve by taking absorbance at 520nm.

Catalase (µmole/g/min FW): Catalase (CAT) activity was estimated according to the reference method [46]. All steps of extraction were carried out at -4°C. Five hundred mg of leaves from transgenic and non-transgenic plants were used.

The leaves were washed with distilled water, filter paper dried and 3 mL extraction buffer (potassium phosphate) containing 0.1 mM EDTA, 1% (w/v) Polyvinylpyrrolidone (PVP, 0.5% Triton X-100 and 20% glycerol. pH was adjusted to 7.8. The homogenate was centrifuged at 10,000xg for 15 min at 4°C. The supernatant was aliquoted and used as crude enzyme extract. The reaction mixture in final volume of 3 mL, contained 0.1M phosphate buffer (pH 7.0), 10mM H_2O_2 and 50µL of cell-free extract. Reaction was initiated with the addition of H_2O_2. Enzyme activity was determined by via degradation of H_2O_2 at 240nm for 2 min. The enzyme activity was calculated using 39.4 $mM^{-1}cm^{-1}$ as the extinction coefficient value of H_2O_2. One unit (1U) of enzyme activity correlated with nmol H_2O_2 consumed during reading.

Peroxidase (µmole/g/min FW): Estimation of peroxidase (POX) activity was by reference method [47]. The extraction was the same as used for catalase. Three ml of reaction mixture contained 0.1 M phosphate buffer (pH 7.0), 0.1 mM guaiacol, 0.1 mM H_2O_2 and 50 µL cell-free extract. Reaction was started with H_2O_2. Absorbance at 470 nm was recorded for 2 min. The activity was calculated using the extinction coefficient value of 22.6 $mM^{-1}cm^{-1}$ for guaiacol. One unit of enzyme activity was equivalent to µmol of H_2O_2 oxidized.

Na^+ - K^+ (ppm) content: 500 mg plant material in a 50 or 100ml conical flask. Add 10 mL of diacid mixture of HNO_3 and $HClO_4$ in a ratio of 4:1 and kept overnight. Keep on a hot plate and heat gently first. Volume is reduced to 3-4 ml Cooled and transferred, diluted to 50mL using a volumetric flask and filtered for further analysis. After digestion, reading was read using a Flame photometer (S-935 Flame photometer) instrument.

Statistical analysis: Statistical analysis was carried out on physiological data recorded in T_2 using two factorial CRD (Completely Randomized Design) test in OPSTAT program [48].

Results

Genomic DNA isolation

The agarose gel electrophoresis showed clear, sharp and intact bands with no shearing (Figure 2).

Molecular Characterization of Transgenic Chickpea Plants Carrying *OsRuv*B Hene

The putative transgenic plants were screened for the presence of *OsRuv*B gene in T_2 generation with the help of PCR using gene-specific primers (Figure 3).

Screening of putative transgenic chickpea plants: An amplified fragment of 557 bp confirmed the presence of the transgene corresponding to the amplified product from plasmid DNA carrying *OsRuv*B gene. Out of 33 plants screened for the presence of *OsRuv*B gene, 12 plants showed a distinct band of 557

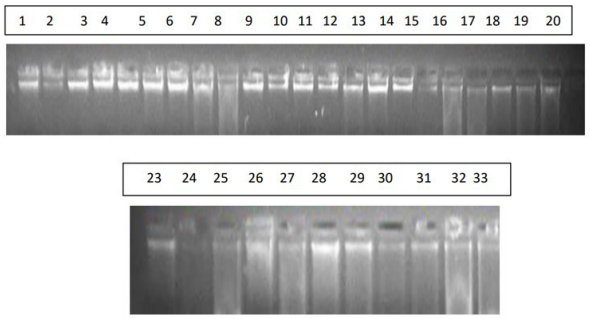

Figure 2: 0.8% agarose gel showing genomic DNA of T_2 chickpea plants 1-33- T_2 chickpea plant genomic DNA.

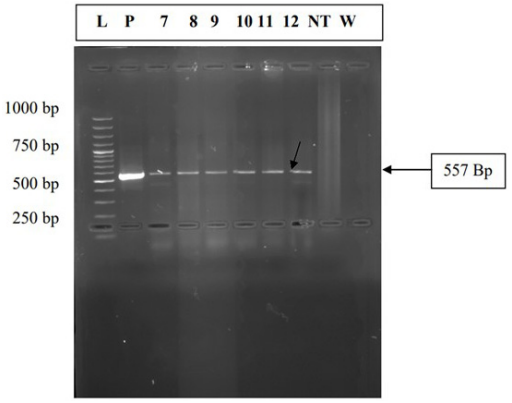

Figure 3: P: 1.5% agarose gel showing amplification of 557 bp fragment of *OsRuv*B gene in T_2 generation plants. P: Positive Control (Plasmid DNA), NT: Non transformed, W: water, 7-12 represent T_2 generation transgenic chickpea plants.

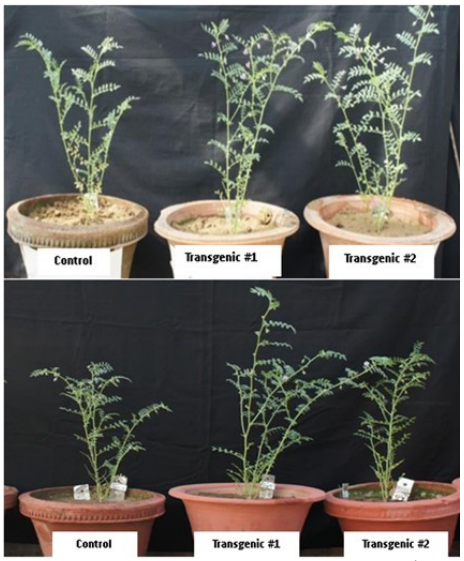

Figure 4: Morphology of plants before stress and 7th day after salt treatment.

Figure 5: Comparison of roots of wild and transgenic types under salinity. There was a clear difference observed in roots of wild and transgenic types under stress condition. Wild type under stress had poor root growth over wild type control.

bp, representing a transformation efficiency of 36.6% (Table 3).

Morpho-Physiological Biochemical Parameters of Transgenic Plants Under Salinity

Morphology: A difference was seen in morphology of wild type and transgenic plants. Wild type plants had lesser growth in terms of shoots while transgenic plants show better growth. Morphology of plants before and 7th day after salt treatment is shown in Figure 4.

Root Morphology: There was a clear difference observed in roots of wild and transgenic types under stress condition. Wild type under stress had poor root growth over wild type control. Transgenic under stress had more branched roots and looks more networks due to *OsRuvB*gene which carries more water absorbance over wild type (Figure 5).

Chlorophyll content (mg/g FW): The chlorophyll content of wild-type and transgenic plants were decreased under salt treatment (100mM). Minimum chlorophyll content was observed in wild type under stress. In wild type under stress there was a 0.7-fold decrease in chlorophyll content with respect to wild type control in normal conditions). We also observed a 1.23-fold increase in chlorophyll content in line 4 under stress with respect to wild type control. Line 4 transgenic control was also increased 1.29-foldwith respect to wild type control. In line 8, transgenic control there was a 1.6-fold increase of chlorophyll content with respect to wild type control whereas in line 8 transgenic under stress had increased1.4-fold respect to wild type Control. In both transgenic, line 8 was performing better under stress condition in terms of chlorophyll content (Figure 6, Table 4).

Relative water content (%): Relative water content of wild type and transgenic lines were decreased under salt treatment (100mM). The minimum RWC was decrease in wild type under stress when compared with all other plants. There was a 0.7-fold decrease in RWC in wild type under stress condition. Inline 4 transgenic control there was an increase (1.1-fold) of RWC with respect to wild type control Line 4 under stress there was a decrease (0.9-fold) of RWC with respect to wild type control. In line 8 transgenic controls we observed a1.1-fold an increase of RWC and under stress it was 1.07-foldincrease compared to wild type control (Figure 7, Table 4).

Electrolyte leakage (%): Electrolyte leakage was more in wild type plant than the transgenic plant. There was a 1.3-fold increase of electrolyte leakage in wild type stress with respect to wild type control. In addition, line 4 control decreased (0.9-fold) whereas transgenic treated plants were increased by 1.1-fold-compared to wild type control. In line 8, transgenic control decreased 0.9-foldwhereas transgenic treated plants were increased 1.1-fold with respect to wild type control. Overall, line 8 had lesser electrolyte leakage than others (Figure 8, Table 4).

Lipid peroxidation (µmol/g FW): Under stress conditions, lipid peroxidation increased in both transgenic and wild-type plants. The highest lipid peroxidation was observed in wild type under salt stress was increased 1.4-fold compared to wild type control. In line 4 transgenic control, it decreased 0.8-fold while in transgenic treated it was increased 1.2-fold over wild type control. In line 8 transgenic control lipid peroxidation decreases (0.6-fold) whereas in transgenic treated plants it was decrease (0.9-fold) fold. d (Figure 9, Table 4).

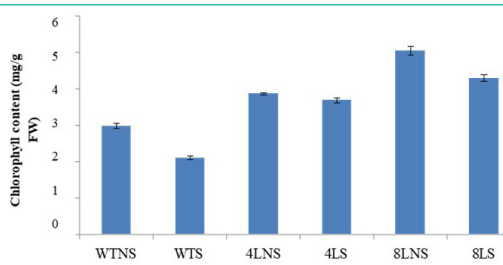

Figure 6: Effect of 100 mM salt stress on chlorophyll content of wild-type and T_2transgenic chickpea plants.

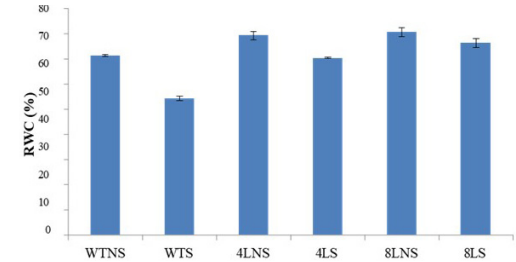

Figure 7: Effect of 100mMsalt stress on relative water content of wild-type and T_2transgenic chickpea plants.

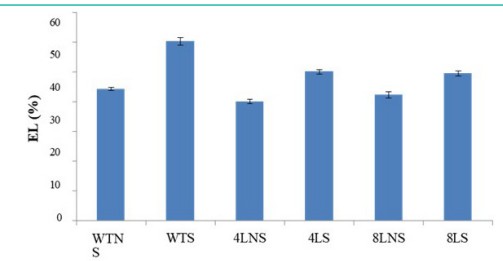

Figure 8: Effect of 100 mM salt stress on electrolyte leakage of wild-type and T2 transgenic chickpea plants.

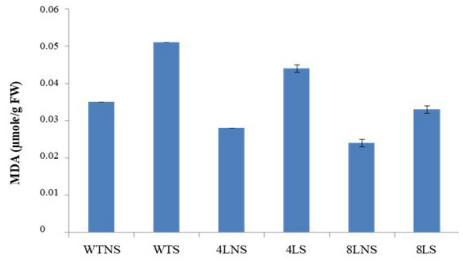

Figure 9: Effect of 100 mM salt stress on lipid peroxidation of wild-type and T2 transgenic chickpea plants.

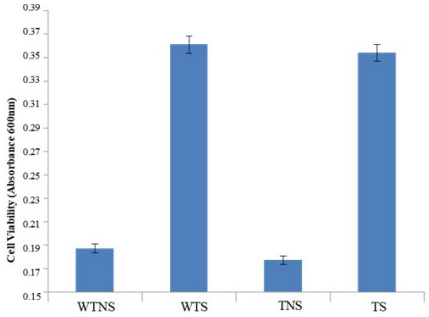

Figure 10: Effect of salinity (100mM) on cell viability of wild and transgenic chickpea.

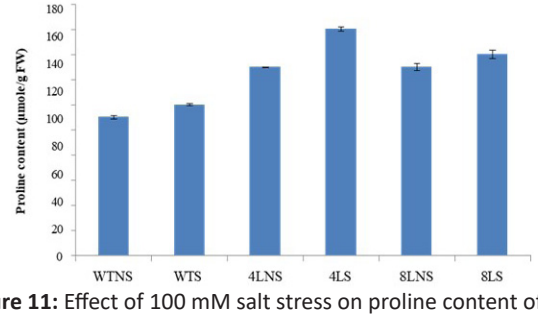

Figure 11: Effect of 100 mM salt stress on proline content of wild-type and T2 transgenic chickpea plants.

Cell viability test: More cell viability was recorded in wild type control plants than wild type treated. Lower absorbance show less damage was observed in wild type, whereas wild type treated show more absorbance suggesting more cellular damage. Similar changes were observed in transgenic line (Figure 10).

Proline content (µmol/g FW): Proline content of both transgenic and wild-type plants increased under stress conditions. The minimum proline content was observed in wild type control while under stress it was increased (1.09-fold). In line 4 transgenic controls were increased (1.3-fold) and treated also increased (1.6-fold) over wild type control. While in line 8 transgenic controls was increased (1.3-fold) and in transgenic treated it was increased (1.4-fold) over wild type control (Figure 11, Table 4).

Catalase activity (µmole/g/min FW): Catalase (CAT) activity of both transgenic and wild-type plants increased under stress conditions. The minimum CAT activity was observed in wild type control. There was a1.1-fold increase in CAT activity in wild type under stress over wild type control. Among both transgenic lines, line 8 had more CAT activity. Line 4 transgenic control had a 1.2-fold increase in CAT activity whereas in transgenic treated it increased 1.37-fold over wild type control. Under stress line 8 had increased 1.3-fold while transgenic control was increased (1.2-fold) over wild type control (Figure 12, Table 4).

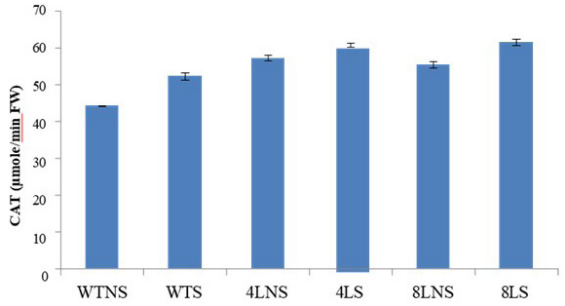

Figure 12: Effect of 100 mM salt stress on catalase activity of wild-type and T_2 transgenic chickpea plants.

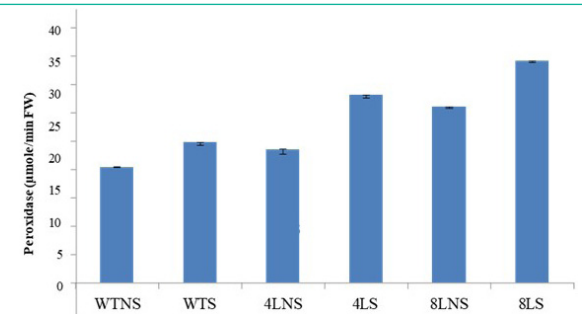

Figure 13: Effect of 100 mM salt stress on peroxidase activity of wild-type and T_2 transgenic chickpea plants.

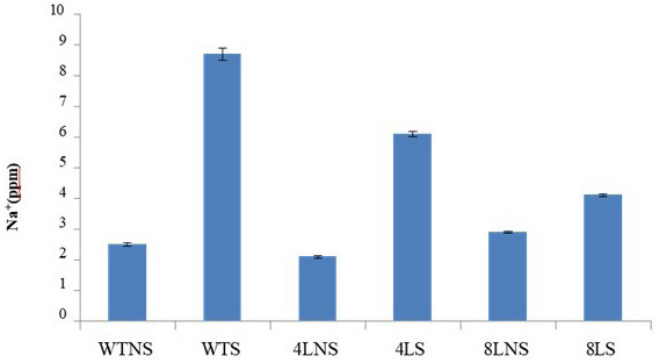

Figure 14: Effect of 100mM salt stress on Na^+ content of wild-type and T_2 transgenic chickpea plants.

Peroxidase activity (µmole/g/min FW): Peroxidase activity in both wild type and transgenic plants increased under stress. The minimum peroxidase activity was observed in wild type control whereas in wild type under salt stress it increased 1.2-fold. Among both transgenic lines, line 4 control increased (1.1-fold) whereas those treated plants increased 1.6-fold compared to wild type control. Line 8 under stress had increased 1.9-fold whereas line 8 control had increased 1.5-fold over wild type control (Figure 13, Table 4).

Na^+- K^+ content: There was an increase of sodium content under salt stress in both wild type and transgenic type while potassium content was more in non-treated in both wild and transgenic type. There was a3.4-fold increase in sodium content in wild type under stress over wild type control. Along with this, line 4, transgenic control sodium levels decreased 0.8-fold, whereas transgenic treated increased 2.2-fold over wild type control. In line 8, transgenic control there was an increase 1.1-fold while in transgenic treated had a 1.6-fold increase over wild type control. While Na+/K+ ratio was higher under stress condition, K+/Na+ ratio is higher under non stress condition (Figure 14, Table 4).

In the case of potassium ion, there was 0.6-fold decrease in wild type under stress over wild type control. In line 4 transgenic control, it was increased by 1.2-fold whereas in treated transgenics, had a 0.5-fold decrease over wild type control. In line 8 transgenic control, a 1.2-fold increase was observed, whereas in transgenic treated it was decreasing0.7-fold over wild type control. Overall, both transgenic lines had considerable Na^+-K^+ balance compared to their respective control (Figure 15-17, Table 4).

Protein study of wild type and transgenic plants: Protein content is increased under stress in both wild and transgenic plants. But it was increased in wild than transgenic. The minimum protein content was observed in wild type control. While the maximum protein content was observed in line 4 under stress. In wild type under stress protein content was increased (1.09-fold) over wild type. Along this in line 4 transgenic control the protein content was increased (1.2-fold) and in transgenic under stress it was increased (1.3-fold) over wild type control. In line 8 transgenic control had increased (1.1-fold) and in transgenic treated it was increased (1.2-fold) with respect to wild type control (Figure 18). The expression level of peptides of ~16, 20, 27, 37 and 66 kDa either showed decreased or increased accumulation with respect to WT. Less accumulation of Rubisco protein (~66kDa) under stress in both the WT and transgenic than their respective control was observed.

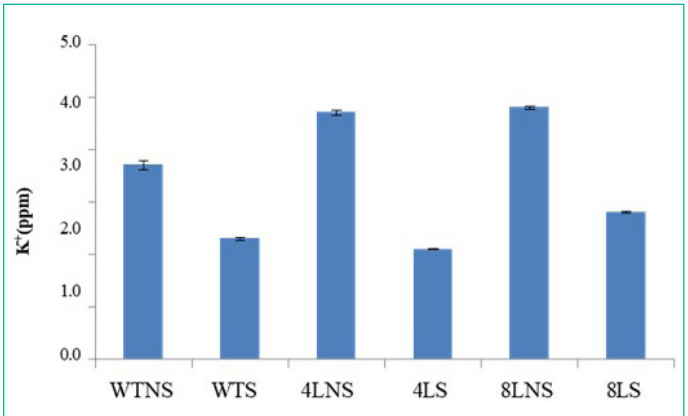

Figure 15: Effect of 100mM salt stress on K^+ content of wild-type and T_2 transgenic chickpea plants.

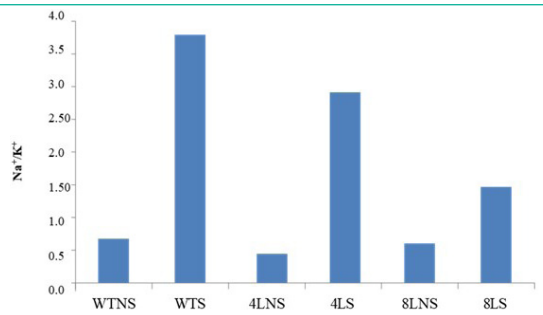

Figure 16: Ratio of Na^+/K^+ ion under stress of wild and transgenic chickpea plants.

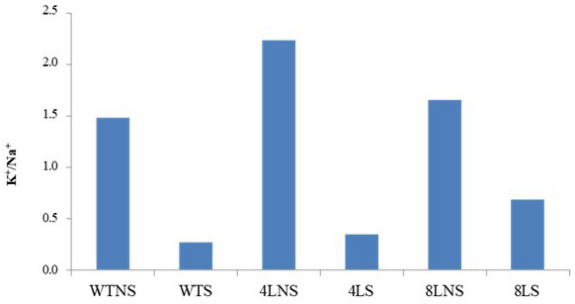

Figure 17: Ratio of K^+/Na^+ ion under stress of wild and transgenic chickpea plants.

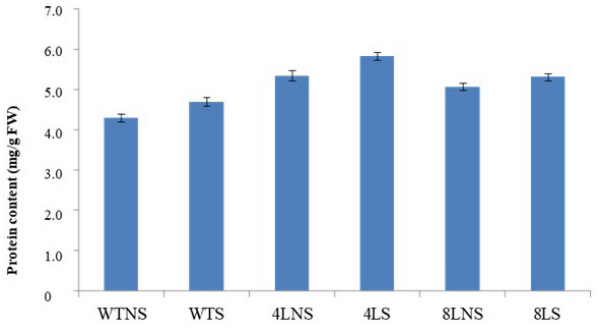

Figure 18: Effect of 100mM salt stress on protein content wild and transgenic types at 7th day after stress.

Discussion

In the present study, putative transformants had been examined for integration of *OsRuvB*in T_2 generation with the use of PCR evaluation with gene-precise primers. Non-transformed plants served as negative control and plasmid DNA served as effective manipulate for screening of transgenics. Of 33 plants screened, 12 plants showed a 557 bp amplicon representing a transformation efficiency of 36.6%.

PCR study was undertaken with the genetic engineering salt-strain tolerance in *V. mungo* [49] via way of means of overexpression of the glyoxalase I (gly I) gene remoted from *Brassica juncea* [50]. Similarly, PCR primarily based screening of purported transgenic has been employed by varied researchers to substantiate the incorporation of the transgene in legumes like *Cicer arietinum* [51], *Medicago sativa* [52], *Arabidopsis thaliana* [53] and *Glycine max* [54].

Physiological biochemical evaluation of T_2 generation plants subjected to 100mM NaCl at flowering stage to assess the transgene efficacy in mediating salt tolerance. Various physio-biochemical parameters such as chlorophyll content, relative water content, electrolyte leakage, lipid peroxidation, Na^+-K^+ content, root morphology, proline content, catalase and peroxidase, cell viability had been recorded.

A clear difference was seen in the morphology of wild and transgenic plants. Wild type plants show lesser growth in terms of growth parameters such as root and shoot while transgenic plants have better growth and development.

The chlorophyll content declined with salinity strain. The reduced chlorophyll content could be explained perhaps by the destabilizing effect of Na^+ ions the thylakoid membrane by disrupting the lipid bilayer and membrane proteins. Chlorophyll content of the wild-type and transgenic plants were decreased under salt treatment (100 mM) than their respective control. NaCl treatment has been attributed to the destruction of chlorophyll pigments and the instability of pigment-protein complex [55-57].

The relative water content is a physiological index that is used to investigate the water retention capacity and serves as a suitable parameter to measure the water status and the osmotic settings of plants under abiotic stress [58]. RWC decreased in both WT and transgenic lines under salt stress, but the decrease was more pronounced in WT plants, suggesting that as the duration of salt stress increased, transgenic lines were effectively able to retain more water in their tissues than WT. The results were comparable to related transformation studies that reported better water retention in in transgenic plants such as corn [59], tomato [60], and tobacco [61] for drought stress [62] and pigeon pea [63] for salt stress.

The degree of cell membrane damage induced by salt stress can be measured by estimating the electrolyte leakage. Electrolyte leakage is considered a physiological indicator of tolerance to salt. In the present investigation the wild and transgenic both showed an increase of electrolyte under stress. But transgenic plants were less damaged over wild type. They were more tolerant under stress conditions than wild type. In a study, a defensin gene, Ca-AFP, from *Cicer arietinum*, was cloned and transformed in *Arabidopsis thaliana* and under simulated water-deficit conditions, the transgenic *A. thaliana* plants had higher accumulation of the Ca-AFP transcript compared to that under non-stress condition and exhibited reduced ion leakage in the transgenic plants as compared to wild-type plants [64].The transgenic peanut plants not only accumulated high levels of solutes, but also showed increased membrane integrity under severe stress conditions. Transgenic peanut lines over-expressing *HDG11* showed significantly reduced electrolyte leakage [65]. Similar results were obtained in soybean over-expressing *MsWRKY11* [66], tobacco over-expressing *p68* [19]. NaCl toxicity, the foremost form of salt in most saline soils, enhances the Na content and therefore influences the absorption of different mineral factors [67]. Differences in the accumulation of K^+/ Na^+ can also be involved in cultivar behavior under salt stress [68] which suggests that toxic ions can accumulate more in genotypes due to increased perspiration. Sensitive varieties accumulate ions more quickly than tolerant and this accumulation of ions leads to the death of the leaves and gradually to the death of the plant [69].

In the present investigation the sodium content was high in stress condition and potassium vice-versa. The maximum sodium content was present in wild type whereas in transgenic lines it accumulated less over wild type and potassium content vice-versa. Overall, the transgenic line imparts better tolerance in ion toxicity than wild type. Similar results were found in pea at different salt treatments where leaf K^+ /Na^+ ratio decreased significantly with increasing salt levels [70]. It has been reported in the youngest fully developed leaves that highest Na^+ concentration was related to a decrease in chickpea yield under salty conditions [71].

Additional evidence for decreased oxidative damage was tested through much less membrane harm index and MDA content inside the transgenic plants as compared to chickpea plants. MDA is an indicator of peroxide membrane lipid, so it is an effective marker of cell damage induced by oxidative stress. Reduce MDA levels to control oxidative stress and reduce the rate of oxidative stress membrane damage It can be concluded from this that the *OsRuvB* transgene plays a key role in maintaining a lower ROS content under salt stress conditions and thus prevents membrane damage in plants. In this study the MDA content was maximum in wild type under stress while transgenic had also increased MDA content but it was low as compared to wild type.

Same trends were observed in pigeon pea have been reported [72] and in rice [73]. Proline is associate degree osmoprotectant that incorporates a key role in maintaining the diffusion balance in crop plants, protective the cell organelles, enzymes and enhancing the osmolarity of the plant cells below stress conditions [72]. In present investigation proline content was increase in both wild and transgenic. Accumulation under salt stress was comparatively lower in wild-type plants as compared to transgenic plants. Among transgenic lower accumulation of proline were observed in line 8 then line 4. However, the increase of proline and total soluble sugars in wild-type plants was significantly higher than that of transgenic plants under stress which indicates that other osmotic compounds such as glycine, betaine or polyamines, etc., not considered in this study, can play a decisive role in osmotic protection [74].

Excessive Reactive Oxygen Species (ROS) formation can induce oxidative stress, leading to cell damage that can culminate in cell death. Therefore, cells have antioxidant (catalase, peroxidase etc.) networks to scavenge excessively produced ROS. Catalase is a heme-containing enzyme that catalyzes the breakdown of H_2O_2 into H_2O and O_2. Catalase eliminates the H_2O_2 generated in peroxisomes by oxidases involved in β-oxidation of fatty acids, photorespiration, purine catabolism and during oxidative stress. A significantly higher catalase activity in transgenic lines of chickpea demonstrated their ability to scavenge ROS. In the present study, CAT was increased in all plants. But more pronounced CAT activity was seen in transgenic line. In transgenic line 4 had more CAT activity which shows greater tolerance under stress. Improved catalase activity had been cited in *Arabidopsis* [75] and peanut [76].

Peroxidase is a heme-containing protein, which oxidizes certain substrates at the expense of H_2O_2 and rid the cellular of excess peroxide produced with the aid of metabolic interest under both regular and pressure conditions. The production of antioxidant enzymes under salt stress enables an efficient management of ROS and therefore improves the life of the cells and their components. In the present study, peroxidase activity was increased in both wild and transgenic types under 100 mM salt stress. POX activity was recorded low in wild while high in transgenic. Among transgenic line 8 had more POX activity. A similar trend was observed in *Sorghum bicolor* [77,78] and tomato [79].

Plant protein pattern is influenced by salinity in two ways. First one it lowers total protein content [80] and second is restricting the production of specific proteins [81] required for tolerating effects of salinity through engaging ABA [82]. Total protein content was higher in both wild and transgenic under stress condition but more in transgenic case.

Confirmed with our findings, enhanced protein content upon salt stress is reported in different tolerant plant species [83,84]. Stress-regulated proteins could be classified into two groups: proteins that take part in signal transduction comprising transcription factors, RNA-binding proteins, protein kinases and phosphatases [85,86] and proteins that might be directly playing role in plant survival under stress conditions. The second group includes proteins involved in ion homoeostasis through increased synthesis of osmolytes and compatible solutes [87] and these are rectified through induction in water channels [88], oxygenic enzymes system [89] and may be some specific protective proteins [90]. Therefore, understanding the molecular details of plant stress response depends on clarifying the biological activity of individual proteins and their interaction with other cellular components. Salt stress proteins of low mass namely 20-24 and 26 kDa have been reported in barley [91,92]. The increase level of 26 and 27 kDa proteins in barley by salt stress [93] and in rice considered to be the acquisition of tolerance to salt stress since the amount of 26 kDa protein concentration of NaCl in the medium [94].

Conclusions

Chickpea is a self-pollinated true diploid (2n=2x=16) winter season leguminous crop that ranks second among food grain legumes in the world after soybean. Presently, it is cultivated in more than fifty countries across the Indian subcontinent, North Africa, the Middle East, southern Europe, USA, and Australia. India is the largest producer of chickpea accounting for 75% of the global chickpea production. Conventional breeding has made numerous attempts to enhance chickpea towards salt stress however no predominant step forward has been taken. Introduction of foreign genes for salt tolerance from primitive landraces and elite cultivars is a dependable opportunity and may be achieved with the assist of genetic engineering.

In the present study, transgenic chickpea plants (var. HC-1) carrying *OsRuv*B gene were screened for salt stress tolerance. The seeds of wild and chickpea were grown in pots in green house. After germination leaves were collected for DNA isolation and run gel electrophoresis for confirmation of DNA. PCR based screening was used for identification of putative transformed plants using OsRuvB gene-specific primers which produced an amplicon size of 557 bp. Out of 33 plants screened for the presence of OsRuvB gene, 12 plants showed a distinct band of 557 bp, representing a transformation efficiency of 36.6%.

Transgenic and wild type T2 chickpea plants were subjected to salt stress (100 mM) after germination at flowering stage and various physio-biochemical parameters like relative water content, chlorophyll content, electrolyte leakage, Na+-K+ content, lipid peroxidation, root morphology, catalase activity, peroxidase activity and proline content were studied.

Salt stress affected the various physio-biochemical parameters resulting in decrease in chlorophyll and relative water content and an increase in electrolyte leakage, lipid peroxidation and proline content. The activity of antioxidant enzymes, catalase and peroxidase increased with salt stress. Sodium content is increased and potassium vice versa.

Among all the transgenic lines, line 8 performed better in terms of various physio- biochemical parameters except ROS in which line 4 performed better studied under salt stress conditions.

From the present study we can conclude that *OsRuv*B coding for DNA helicase in mitigating salt stress in transgenic chickpea plants. Field studies are required to further confirm the effectiveness of the chickpea transgenic plants in the real saline situations.

Author Statements

Author Contributions

Conceptualization, N.L. and D.K.; methodology, D.K.; validation, N.L., and A.Z.; formal analysis, D.K; P.S.; investigation, D.K; and. N.L.; resources, A.Z.; data curation, D.K.; and P.S. writing—original draft preparation, D.K; N.L; P.S; and A.Z; writing—review and editing, A.Z; N.L; and P.S.; visualization, D.K.; supervision, N.L; project administration, N.L. All authors have read and agreed to the published version of the manuscript.

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