MATERIALS AND METHODS

3.1 Screening of mungbean germplasm for salt tolerance

Mungbean germplasm was screened for salt tolerance 1) In vitro at seed germination and early seedling growth level in petridishes and 2) In vivo at later developmental stages in pots containing soil.

3.1.1 Plant material

Seeds of 117 genotypes of mungbean and twenty two lines of wild relatives of mungbean were procured from core collection at National Bureau of Plant Genetic Resources (NBPGR), New Delhi and Division of Genetics, Indian Agricultural Research Institute, New Delhi (Tables 3.1 and 3.2). The selected genotypes marked with asterisk (*) and the wild relatives were screened for salt tolerance by pot-trial method up to harvest of the crop.

3.1.2 Saline Treatments

A total of five different salinity levels were prepared as EC0, EC4.0, EC7.0, EC10.0, and EC16.0 (dS/m) for imposing salinity stress during germination and early seedling growth stage in petridishes. The saline solutions with different electrical conductivity were prepared from mixture of salts (NaCl, Na2SO4, CaCl2, and MgSO4) in defined proportions i.e. Na: Ca, Mg (2:1) and Cl: SO4 (3:1) for EC = 1.0 dS/m. Three salinity levels of 0mM NaCl (Control) , 50mM NaCl (T1), and 75mM NaCl (T2) and two salinity levels of 0mM NaCl (Control) and 250mM NaCl were prepared by dissolving sodium chloride in the water used for irrigation for the screening of cultivated and wild relatives of mungbean in pots respectively. The control treatment was without sodium chloride.

3.1.3 Evaluation of salt tolerance at germination and early seedling growth

Initially, seeds of 117 accessions of mungbean were allowed to germinate on filter paper moistened with 10 ml of saline solutions of different concentrations i.e. EC4.0, EC7.0, EC10.0, and EC16.0 (dS/m) along with their respective control (EC0) in petriplates consisting of 5 seeds /genotype /treatment. The experiment was carried out with four replicates per salinity treatment at mean temperature of 28 ± 2°C and with the relative humidity more than 65%. The petridishes were tightly sealed with parafilm to prevent evaporation of water thus minimizing changes in concentration of salt solutions and placed under 16 h photoperiod of cool white-fluorescent light of intensity 80 uEm²s⁻¹ for
seed germination. The germination percentage, seedling growth characteristics (radical, plumule and total seedling length), and seedling vigor were measured on 4th day after germination. All the above said observations were mean of four replications per treatment.

3.2 Screening for salt tolerance at later growth stages up to harvest

Selected genotypes were screened by pot-trial method in complete randomized-block design by observing greater number of more reliable parameters for salt tolerance up to the harvest of the mungbean crop during July to September of 2008, 2009, and 2010.

3.2.1 Sowing of the seeds and salinity stress

Rhizobium treated seeds of all the selected mungbean genotypes were sown in 30 cm earthen pots (30 x 30 cm) containing 10 kg of soil, sand, and manure in 1:2:1 ratio, respectively, during kharif seasons of 2008-2010. The pots used for salinity treatments were lined with 400 gauge polythene bags to avoid leaching of the salt during irrigation. The whole experiment was conducted in pot culture under an artificial rain shelter or hut made up of bamboos and polythene (PVC) with approximate 99% transparency or visibility so that the plants could absorb the sufficient light for photosynthesis and growth and the other contaminating or stress causing factors like natural rain, strong wind etc. interfering with the salinity treatment could be avoided (Figure 3.1). The plants were sprayed with rogor and melathione to control red mites. The removal of the weeds was done by hand regularly and the irrigation practice was maintained manually at regular intervals of time for the crop season. The plants were thinned to 5 plants per pot after one week of seed germination (10 DAS). The NaCl solutions of two concentrations i.e. 50mM (T₁) and 75mM (T₂) was applied to the plants i.e. 2.5 litre/kg of soil, after the emergence of fully expanded primary leaves in all the genotypes (15 DAS) for imposing salinity stress. The plants applied with equal volume of water were used as control (C). Similarly, wild relatives (except control plants) were subjected to high salt stress caused by 250mM NaCl. Scheduled routine of irrigation was practiced for both the control and the salt treated pots throughout the crop growth period.
3.2.2 Methodology used

The effect of salt stress on growth parameters, physiological and biochemical characteristics and yield attributes was measured at different stages of the crop i.e. 1) vegetative stage (15 DAT), 2) 50% flowering stage (30 DAT) and 3) 50% pod filling stage (45 DAT). All the observations were taken with 3 replications per treatment. Survival % was also measured at regular intervals of time after every 15 days after treatment. The leaves samples for RWC, MSI, and total chlorophyll contents were collected early in the morning (6:00 – 7:00 A.M.) from the second fully expanded trifoliate from the top freshly during each growth stage. The leaf samples were brought in triplicates to the laboratory in ice bouquet so that the loss of moisture can be minimized. The soil samples were also collected with 4 replications at different stages for the measurement of EC (units in dS/m) by Conductivity Bridge and pH by pH meter as per the method of Jackson, (1973).

3.2.3 Growth Parameters:

Plant height – root length and shoot length, dry weight of root, shoot, and leaves and leaf area were measured. The samples i.e. root, stem, and leaves were dried at 80°C completely in hot air oven (NSW, New Delhi) for 2 days till constant weights were obtained and then incubated in desiccators before measuring the dry weight. Leaf area was estimated by using Leaf Area meter model Li 3100 (LiCoR Inc. Lincoln, Nebraska, USA). Photosynthesis was measured at 50% flowering stage by Infrared Gas Analyzer (IRGA).

3.2.4 Relative water content (RWC)

Leaf relative water content (RWC) was estimated by recording the turgid weight of 0.5 g fresh leaf samples by keeping in water for 4 h, followed by drying in hot air oven till constant weight is achieved as per the method of (Weatherley, 1950).

\[
RWC = [(\text{Fresh wt.} – \text{Dry wt.}) / (\text{Turgid wt.} – \text{Dry wt.})] \times 100
\]

3.2.5 Membrane stability index (MSI)

Membrane stability index (MSI) was estimated as per Sairam et al., (1997). For the estimation of membrane stability index 100 mg leaf material, in two sets, is taken in test tubes containing 10 ml of double distilled water. One set is heated at 40°C for 30 min
in a metabolic water bath and the electrical conductivity of the solution is recorded on a conductivity bridge (C₁). Second set is boiled at 100°C on a boiling water bath for 10 min and its conductivity is measured on a conductivity bridge (C₂). Membrane stability index (MSI) is calculated as:

\[ MSI = [1 - (C_1 / C_2)] \times 100 \]

### 3.2.6 Chlorophyll contents and Carotenoids

Chlorophyll content was estimated by extracting 0.05 g of the leaf material in 10 ml dimethylsulfoxide (DMSO) as per the method of Hiscox and Israelstam, 1979). Samples were heated in an incubator at 65°C for 4 h and than after cooling to room temperature, the absorbance of extracts were recorded at 663nm and 645nm. Chlorophyll content was calculated as per the formula given by Arnon, 1949.

Chl a: \[12.7 \times A_{663} - 2.69 \times A_{545}\]
Chl b: \[22.9 \times A_{645} - 4.68 \times A_{663}\]

Total chlorophyll = \[20.2 \times A_{645} + 8.02 \times A_{663}\] x V/W x 1000

The values thus obtained were in ug/ml of extract (Solvent). Values in mg/g fresh wt. were obtained by multiplying the above values with “v/w x 1000,” where V is volume of extract; W is fresh wt. of sample. The value of total carotenoids (mg g⁻¹) was determined as per the formula of (hichtenthaler and Wellburn, 1983).

Carotenoids = \[1000 \times A_{470} - (3.27 \times \text{chl a} + 104 \times \text{chl b})\] / 229

### 3.2.7 Proline

Proline is an important imino acid found in proteins, though generally it is referred as an amino acid. Proline content was measured as per the method of Bates et al., (1973). Leaf sample (0.5g) was ground in 10 ml of 3% aqueous sulfosalicylic acid and filtered through Whatman # 2 paper. Addition of 2 mL of acid-ninhydrin and 2 mL of glacial acetic acid was done to 2 mL of this filtrate in a test tube. Heating was done at 100°C in a water bath for 1 h and the reaction was stopped by placing the tubes in an ice bath. Toluene (4 ml) was added to the mixture and vortexed for 15 to 20 seconds. The chromophore was aspirated from the aqueous phase and absorbance of toluene phase was recorded at 520 nm. A blank (minus sample extract) was also run, the absorbance of which was subtracted from sample absorbance and a standard graph was prepared using L-proline as a standard.
3.2.8 Estimation of potassium and sodium

3.2.8.1 Digestion of plant samples

The plant samples were dried in oven at 65 ± 5°C and ground thoroughly by a wiley mill. A representative ground plant sample (0.5g) was taken for digestion. The samples were soaked overnight with 10ml of concentrated HNO₃ in conical flasks (100ml capacity) for pre-digestion and finally digested in a di-acid mixture (20ml) containing HNO₃ and HClO₄ acid (9:4) on digestion unit (Gerhardt Turbothermn). The digested material was cooled, diluted with distilled water and filtered through Whatman No. 42 filter paper. The volume was made up to 25 ml/40ml and stored in a polypropylene container (100ml capacity) for further analysis.

3.2.8.2 Estimation of potassium, sodium and their respective ratio (K⁺/Na⁺)

The K and Na content in the standard solutions and plant samples (leaf, stem, and root) were estimated by using K and Na - specific filters in a flame photometer (ELICO CL361). By plotting a standard curve with known concentration of K and Na, the content of K and Na were calculated in different plant parts. The K/Na ratio was calculated in all the plant samples by dividing their respective individual values.

3.2.9 Yield attributes:

Total number of pods/replication, number of pods/plant, pod length/replication, number of seeds/pod, hundred seeds weight and yield /plant were recorded and yield susceptibility index (%) was calculated under both salinity treatments over control.

3.3 Observations recorded during screening of wild relatives of mungbean

The effect of salt stress on the plant growth, leaf size and color, symptoms of necrosis, chlorosis, and yellow mosaic disease, was observed visually and the comparison of control and salt treated plants was done by photographs only. Finally, the grain yield per plant was measured and compared in non-stressed and stressed conditions.

3.4 Statistical analysis

The data obtained in this study was subjected to analysis of variance (ANNOVA) appropriate to the experimental design. F-test was carried out to test the significance of the treatment differences and the least significant difference (LSD) was computed to test
the significance of different treatment at 5% level of probability by using OPSTAT program, HAU, Hisar.

3.5 Assessment of inherent genetic variability in selected mungbean genotypes for breeding

Out of the cultivated genotypes identified for salt tolerance on the basis of growth and yield characteristics under salinity stress, three salt tolerant and five salt susceptible genotypes were selected for the detection of genetic variability (Table 3.3).

Table 3.3 List of the selected mungbean genotypes used in this study (* and ** showed most salt susceptible and most salt tolerant genotype)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Accessions Name and code used in the study</th>
<th>Type</th>
<th>Total</th>
<th>Origin</th>
<th>Resource</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PLM-884 (P16), PLM-380** (P17), PLM-184 (P19)</td>
<td>Salt tolerant</td>
<td>3</td>
<td>India</td>
<td>NBPG, New Delhi-12</td>
</tr>
<tr>
<td>2</td>
<td>IC-10492 (P1), LGG-450 (P2), PLM-32* (P3), PLM-688 (P4), PLM-775 (P5)</td>
<td>Salt susceptible</td>
<td>5</td>
<td>India</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

3.5.1 Microsatellite markers (SSRs)

A set of 106 SSR primer pairs (sequences available at National Center for Biotechnology Information website) developed from azuki bean [Vigna angularis (Willd.) Ohwi and Ohashi] were tested for PCR amplification and polymorphism to measure the transferability and their utility to assess the genetic variation in the cultivated mungbean accessions selected for salt tolerance and susceptibility (Table 3.4).

3.5.2 DNA extraction and PCR amplification

Seeds of all 8 accessions were allowed to germinate on filter paper soaked with sterilized distilled water in Petri-dishes at 37°C in the incubator under light intensity of 80 u Em⁻²s⁻¹. Total genomic DNA was extracted from primary leaves of one-week old single seedling of each accession by using the ‘Mag Extracter’ plant genome purification Kit (Toyobo, Japan). The quantification of DNA was assayed on 1.0 % agarose gel stained by ethidium bromide against a ladder of known concentrations (50ng and 10ng) of lambda DNA. The PCR was performed by preparing the reaction set up of 5µl volume containing 20ng of template DNA, 10x Buffer with MgCl₂ in final concentration of 1x,
5µM of each forward and reverse primer, 2.5mM of dNTPs and 0.025 units of Taq DNA polymerase (5U/µl). The reaction mixture was run on a thermo cycler and the programme had the following cycle: initial denaturation at 94°C for 30 seconds followed by 40 cycles each consisting of denaturation at 94°C for 30 sec., annealing at 60°C for 1 min., elongation at 72°C for 1 min. Final extension set up was carried out at 72°C for 10 min and the PCR product was covered and kept at 4°C. The PCR products were further diluted 10 times in MQ water as necessary to prevent off-scale fluorescent signals.

3.5.3 Genotyping

A volume of 1 µl of PCR product or 1/10 diluted PCR product was mixed with 9 µl of Hi-Di formamide containing 0.1 µl of GeneScan 500 LIZ size standards (Applied Biosystems). The mixture was denatured at 95°C for 5 min then placed immediately on ice. The denatured products were run on an automated capillary DNA sequencer (ABI Prism3100 Genetic analyzer) which is very sensitive to a little amplified PCR product. Size of SSR fragments was determined with Gene Mapper Ver. 3.0 (Applied Biosystems).

3.5.4 Polyacrylamide Gel Electrophoresis (PAGE)

Additionally, the PCR amplified products were subjected to polyacrylamide gel electrophoresis (PAGE) in 1x TBE for 3 hours. The DNA ladder of 100bp (NEB) with concentration of 50ng/ul was used as molecular weight marker. The ethidium bromide was used for staining the gel which was visualized under UV and photographed using Gel Documentation System.

3.5.5 Analysis of data

The marker data obtained from the sequencer was analyzed by Genemapper software Ver. 3.0 (Applied Biosystems). The amplification of each PCR product was scored across the lanes comparing their respective molecular weights. On the basis of the SSR data obtained with all polymorphic primers, the DNA of the following combinations of genotypes were subsequently analysed:
The genotypes were paired, i.e. one salt tolerant vs. all salt susceptible for the measurement of polymorphism and selection of the desired combinations to be used as parents in breeding for salt tolerance. Polymorphism information content (PIC value) was calculated for each SSR locus by the algorithm following (Weir, 1996): PIC = 1-(∑pi²), where, 1 is the total number of the alleles detected for a SSR marker and pi is the frequency of the ith allele in selected 8 parental genotypes of mungbean undertaken in this study. The data matrix of genotypes x alleles for a locus were used to calculate Jaccard similarity coefficients which were subjected to cluster analysis using NTSYS-pc version 2.1.1.2 (Exeter Corporation, USA). Principal component analysis was also performed using the same software on the basis of similarity coefficients.

3.6 Development of gene-specific microsatellite markers (SSRs) for mungbean and their utility in genetic analysis

The sequence of the candidate genes involved in salt overlay sensitive (SOS) pathway available for model plants and related legumes was searched in the GenBank at NCBI database. The sequence obtained for the sos genes was further searched for homology by BLAST at NCBI. The highly homologous sequences (Table 3.5) were aligned by Multiple Sequence Alignment Tool to find out the conserved regions for the genes (sos1, sos2, sos3, and nhx-1) which were further searched for the presence of Simple Sequence Repeats (SSRs) by using the WebSAT software (WS Martins, 2009) and the SSR primers were designed flanking the simple sequence repeats by the similar software. The details of the 38 SSR primers used in this study is presented in (Table 3.6).

3.6.1 Growing of parental genotypes of mungbean

Seeds of the selected twelve salt resistant and susceptible parental genotypes (cultivated and wild relatives) of mungbean with genetically diverse background and different adaptations to salinity stress were sown in field plot of size 30’x20’ at Herbal
Garden, Maharshi Dayanand University, Rohtak, Haryana, during March-May, 2012 (Table 3.7).

Table 3.7 Details of the selected mungbean genotypes used.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of the genotypes</th>
<th>Salt response</th>
<th>Total no. of genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IC 10492a, PLM 32a, IC 2056-2, K 851a, BB 9 2Ra (<em>V. Sublobata</em>)</td>
<td>Salt susceptible</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>PLM 562b, PLM 380b, WGG 37b, IC 615-5c, PLM 891c, TCR 86b (<em>V. trifolobata</em>), EC 528960 (<em>V. luteola</em>)</td>
<td>Salt resistant</td>
<td>7</td>
</tr>
</tbody>
</table>

*as wild relatives of mungbean selected for breeding, a = most salt sensitive genotypes and b = most salt resistant genotypes under high salinity, and c = salt resistant genotypes under low salinity

3.6.2 DNA Extraction and Quantification

Total genomic DNA was extracted from the young leaves (20 days old) of all the 12 salt resistant and salt susceptible parental genotypes of mungbean used in this study using Gene Elute Plant genomic DNA Extraction Kit (Sigma) by following manufacturer’s instructions. The quantification of DNA was assayed on 0.8% agarose gel electrophoresis in 1x TAE for half an hour against the ladder of known concentration (100ng/ul) of lambda DNA.

3.6.3 PCR analysis using SSRs primer pairs

Prior to PCR amplification the DNA samples were diluted (30 ng/µl). The reaction was prepared in 20 µl volume containing 30 ng of template DNA, 10X Taq buffer B (Bangalore Genei, India) in final concentration of 1X, each forward and reverse primer (2.5µM, Sigma), 2.5 mM of dNTPs (Fermentas), 25mM MgCl₂ (Banglore Genei) and 0.03 units of Taq DNA polymerase (5U/µl, Banglore Genei) and run on thermo
cycler (Applied Biosystem Gene AMP PCR System 9700). The PCR programme used for SSRs amplification was consisted of initial denaturation at 94°C for 45 seconds followed by 38 cycles each consisting of denaturation at 94°C for 30 sec., annealing at 50 - 60°C for 1 min., elongation at 72°C for 30 seconds. Final extension set up was carried out at 72°C for 10 min and cooling at 4°C for infinite. PCR amplified products were resolved on 3.0% agarose gel electrophoresis in 1x TAE for two and half hour at constant power supply (50V/cm). The 100bp DNA ladder (Fermentas) with concentration of 50ng/ul was used as molecular weight marker. The gel was stained with ethidium bromide, visualized and photographed using Gel Documentation System (CFW- 1312M, BioRad).

3.6.4 Statistical analysis

The reproducible DNA bands specific to each primer set were scored manually in all genotypes based on positions of the bands relative to the known molecular weight ladder sequentially. Only clearly visible bands were recorded as 0 or 1 for their absence and presence respectively in the data matrix and diffused bands considered as missing data were avoided during scoring. Null allele was assigned to the genotype for the SSR locus whenever the amplification product was difficult to detect for a particular genotype-marker combination. The binary data matrix of genotypes x alleles was subjected to an unweighted pair group method with an arithmetic mean (UPGMA) cluster analysis by NTSYS-PC version 2.1.1.2 software (Exeter Corporation, USA). The principal component analysis (3-D plot) was also performed on the basis of Jaccard similarity coefficients (J-values) using the similar software in support of dendrogram. Polymorphism information content (PIC) values for each SSR locus was calculated according to the algorithm following Anderson et al. (1993) as PIC= 1-(Σp^2). The resolving power (Rp) for each primer was calculated as Rp = ΣIb, where Ib [band informativeness] takes the value: [1 - (2 × (0.5 - p)], and p is the proportion of the genotype of different Vigna species containing that band (Prevost and Wilkinson, 1999). Marker index of each SSR primer was calculated using average diversity index (Dlav) as MlDl = Dlav x EMR where Dlav = 1-Σp^2 and Effective multiplex ratio (EMR) = np,β which is product of number of polymorphic alleles (np) and fraction of the polymorphic markers (β).

3.7 Breeding for salt tolerance in mungbean
The identified salt tolerant and sensitive mungbean genotypes (cultivated and wild relatives) were crossed in the experimental field at herbal garden during March-June, 2012. The genotype JP31300 was selected as salt tolerant cultivar by Sunil at National Institute of Agrobiological Sciences, Japan was also included in this study (Table 3.8).
Table 3.8 Details of the mungbean genotypes used in breeding for salt tolerance

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of the genotypes</th>
<th>Salt response</th>
<th>Type of hybridization involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IC 10492(^{a}), PLM 32(^{a}), IC 2056, K 851(^{a}), BB 9 2R(^{a}) (V. Sublobata)*</td>
<td>Salt susceptible (♀)</td>
<td>Open</td>
</tr>
<tr>
<td>2</td>
<td>PLM 562(^{b}), PLM 380(^{b}), WGG 37(^{b}), JP31300(^{b}), TCR 86(^{b}) (V. trilobata)<em>, EC528960(^{b}) (V.luteola)</em></td>
<td>Salt resistant (♂)</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

*as wild relatives of mungbean, a = most salt sensitive genotypes, and b = most salt resistant genotypes

The different types of crosses involved in this study were as follows:

\[ \text{Female parent (♀)} \times \text{Male parent (♂)} \]  

<table>
<thead>
<tr>
<th>Experimental Place involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Salt susceptible (C) x Salt resistant (W) \hspace{1cm} At Herbal Garden, MDU, Rohtak, (2012)</td>
</tr>
<tr>
<td>2. Salt susceptible (C) x Salt resistant (C)</td>
</tr>
<tr>
<td>3. Salt susceptible (W) x Salt resistant (C)</td>
</tr>
</tbody>
</table>

3.7.1 Method involved

The parental lines of mungbean were grown in experimental field plot (20’x30’) with proper spacing between rows (Figure 3.2). The technique used in crossing depends upon the size of the flower and receptivity of the stigma which varies from crop to crop whether it is self-pollinated or cross-pollinated to gain the better outcome. The selected salt tolerant and salt susceptible genotypes were crossed using hand emasculation and pollination technique (Figure 3.3). The stigma of mungbean which is a self-pollinated crop, is highly receptive in early hours of the day (upto 9:00 a.m. during summer). The emasculation was done in the evening and pollination step in the next morning and both the steps were also performed in early morning hours between 5:00 to 9:00A.M. before the anthers were mature and the stigma would become more receptive which minimizes accidental self pollination. The flower likely to be open next morning of the salt susceptible genotype (♀) was held softly between thumb and forefinger of the left hand.
and genetally opened with the help of fine needle. The upper corolla was kept aside and the keel was opened next and the anthers were removed smoothly without any damage to the receptive and blistering stigma. After effective emasulation, the mature anthers were collected from the salt resistant genotype (♂) and dehisced on the receptive stigma of the female parent for pollination. After that the flower bud was closed properly and covered with very thin layer of cotton to avoid contamination, and the crossed flower was tagged for its identification. The other young flowers were removed closely located to the crossed flower to avoid confusion in the identification of crossed pods. The crossed pods containing the F₁ hybrid seeds were identified, harvested and stored carefully after complete drying. Due to high percentage of flower shedding in mungbean, large number of flowers were tried to make cross for each cross involved in this study. Each salt tolerant genotype (♂) was tried to make a cross with every susceptible genotype (♀) either cultivated or wild relatives of mungbean (open hybridization).

\[
\begin{align*}
\text{IC 10492} & \quad \text{PLM 32} \\
\text{IC 2056-2} & \quad \text{K 851} \\
\text{BB 92R} & \\
\{♀\} & \quad \times \quad \text{PLM 562, PLM 380, WGG 37, JP31300, EC 528960, TCR 86 (♂)}
\end{align*}
\]

a) Salt susceptible genotypes  b) Salt resistant genotypes

A total of 30 type of crosses (shown above) were tried to make to develop the F₁ hybrids by inter-specific and intra-specific hybridization for the genetic improvement of mungbean for salt tolerance because each genotype have some specific features which attract them to include in breeding like high yield with bold seed size in non-stressed conditions or popular variety.

3.8 Confirmation of the true F₁ hybrids using microsatellite markers (SSRS)

The experiment for the confirmation of purity of F₁ hybrids of mungbean developed for salt tolerance was conducted at the Centre for Biotechnology, Maharshi Dayanand University, Rohtak, India during July-September, 2012.

3.8.1 Plant material
The seeds of 21 intra and inter-specific F₁ hybrids of mungbean developed for salt tolerance in our earlier experiment and their respective parental genotypes were used as plant material in this study (Table 3.9 and 3.10).
3.8.2 Microsatellite markers
The microsatellite markers (SSRs) developed from the conserved regions of the candidate genes (sos1, sos2, sos3, and nhx1) of the salt overly sensitive (SOS) pathway, and the azukibean specific SSRs showing polymorphism between the respective parental lines were used to test the purity of all the F1 hybrids. The sequence of the primer pairs used for the confirmation of purity of all the F1 hybrids of mungbean from 5’ end to 3’ end along with their respective melting temperature (Tm) and product size is given in Table 3.11.

3.8.3 Raising of F1 hybrids of mungbean along with their respective parents
Seeds of all the intra and inter-specific F1 hybrids of mungbean from each F1 pod along with their respective parents were sown in earthen pots (10 inches) having sand, soil and farmyard manure (1:2:1 ratio) respectively in the green house and in experimental field at herbal garden, CBT, M. D. U. Rohtak during July-September 2012.

3.8.4 DNA Extraction and quantification
Total genomic DNA was extracted from the young leaves of all the F1 plants / cross type (25days old) and of the respective progenitors (P1- P10) using Gene Elute Plant genomic DNA Extraction Kit (Sigma) by following manufacturer’s instructions. The quantification of DNA was assayed on 0.8% agarose gel stained with ethidium bromide against a ladder of known concentrations (100ng) of lambda DNA. After running for about 30min, the gel was removed, visualized and photographed on the Gel documentation System (CFW- 1312M, BioRad).

3.8.5 Polymerase Chain Reaction
The DNA samples were then diluted to 30ng/ul prior to PCR amplification. The PCR was performed by preparing the reaction set up of 10µl volume containing 30ng of template DNA, 10X Taq Buffer B (Banglore Genei) in final concentration of 1X, 5µM of each forward and reverse primer (Sigma), 2.5mM of dNTPs (Fermentas) and 0.025 units of Taq.DNA polymerase (5U/µl, Banglore Genei). The whole reaction set up was run on thermo cycler (Applied Biosystem Gene AMP PCR System 9700) and the programme used was consist of initial denaturation at 94°C for 30 sec followed by 40 cycles each
consisting of denaturation at 94°C for 30 sec., annealing at 55-60°C for 1 min., elongation at 72°C for 30 seconds. Final extension set up was carried out at 72°C for 10 min and cooling was done at 4°C for infinite. The products of these PCR using trait specific SSRs markers were resolved on 3.0% agarose gel electrophoresis in 1x TAE for two and half hours at 50V with constant power supply. The DNA ladder of 100bp (Fermentas) with concentration of 50ng/ul was used as molecular weight marker. The ethidium bromide was used for staining the gels which were documented using Gel Documentation System (CFW- 1312M, BioRad).

3.8.6 Other features observed for the F₁ hybrids

The F₁ hybrids were also observed for their morphology especially root characteristics, growth performance, and behavior of flowering (synchronous or non-synchronous), along with pod length and compared to their respective progenitors during this study.

3.9 Details of the methodology used

Raising of F₁ hybrids along with their respective parents

↓

DNA extraction and quantification on 0.8% agarose gel

↓

Amplification of F₁ DNA and their respective parental genotypes with gene-specific SSRs using PCR

↓

Amplified PCR products run on 3.0 % Agarose gel electrophoresis

↓

Visualization and photographed of the gel on Gel documentation system

↓

Testing of the F₁ hybrids by observing the banding pattern of the SSRs used with respect to the parents
3.10 Backcrossing

The $F_1$ hybrid plants were tried to make a cross with the recurrent parent (backcross) to develop the $BC_1F_1$ population during July-September, 2012 in the field plot at herbal garden, M. D. University, Rohtak, India using hand emasculation and pollination technique, so that the desired trait i.e salt tolerance can be transferred to all the plants of the recurrent parent (salt susceptible).

3.11 Collection of $F_2$ populations

The seeds produced from each $F_1$ hybrid seed per cross type was collected as $F_2$ population separately and stored properly at 4°C for further study in near future.

3.12 Identification of candidate gene(s) of salt overly sensitive pathway and vacuolar antiporter regulating ionic homeostasis under salt stress

3.12.1 Plant material

The salt tolerant genotype PLM380 was further investigated for identification of candidate genes (sos1, sos2, sos3, and nhx1) involved in cellular signaling pathway maintaining ionic balance under salt stress. The genotype was selected due to its high crossing ability, good adaptability under salt stress as high photosynthetic rate and root-shoot ratio, high root density with more secondary branches and greater number of nodules, high $K^+/Na^+$ ratio governing salt tolerance behavior, and high yield stability index under salt stress. The study was conducted at the Centre for Biotechnology, M. D. University, Rohtak, India during Novembre, 2011-January, 2013.

3.12.2 Primers for the candidate gene(s)

The nucleotide sequence of the candidate gene(s) of salt overly sensitive (SOS) pathway available for model plants (Arabidopsis thailiana) or related leguminous family (Glycine max) including mungbean were retrieved from GenBank data base of NCBI and subjected to BLAST for sequence homology with mungbean. Primers were designed from nucleotide sequences of Glycine max (sos1, sos2), Arabidopsis thaliana (sos3) and mungbean (nhx1) manually and using Primer 3 online tool (version 4.0). The details of the primers used for the candidate gene(s) of SOS pathway along with the plant source are given in Table 3.12.
3.12.3 Salinity treatment for total RNA expression study and sampling

Sodium chloride was dissolved in irrigation water to prepare salt solution of 50mM NaCl concentration (EC ~ 5.0 ds/m). Salinity treatment was given to the 21 days old plants (days after germination, DAG) at 2.5 L/kg of soil for 24 hours. Roots and leaves samples from control and salt treated mungbean plants after 24 hours of salt treatment. The sampling was done early in the morning (7:00-7:30 a.m.), samples were brought to the lab in ice bouquet and proceed immediately for RNA extraction.

3.12.4 Total RNA extraction and agarose gel electrophoresis

Total RNA was extracted from the control and salt treated samples using RNeasy Plant Mini Kit (Qiagen) by following the manufacturer’s instructions. The RNA samples were resolved on agarose gel (1.2%) electrophoresis at constant power supply of 70 volts. Ethidium bromide (10mg/ml) stained gel was visualized on UV transilluminator and documented on gel documentation system. The changes and variations in total RNA expression due to salinity stress treatment among all the selected mungbean genotypes were observed by comparing with their respective controls.

3.12.5 RT-PCR analysis and sequencing

Synthesis of cDNA from 1 µg of total RNA of salt resistant cultivar PLM380 was done, and amplification was carried out using the gene-specific primers for the candidate genes [nhx7or sos1, sos2, sos3 (in roots), and nhx1 (in leaves)] using one step RT-PCR Kit (Qiagen). The whole reaction set up was run on thermo cycler (Applied Biosystem Gene AMP PCR System 9700). The thermal conditions consisting of reverse transcription for 30 min at 50°C, initial PCR activation step for 15 min at 95°C, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 52-57°C (vary with each primer set) for 1 min., elongation at 72°C for 1 min. Final extension was carried out at 72°C for 7 min and cooling was done at 4°C for infinite. The amplified PCR products were resolved in 1% agarose gel at 100 V in 1X TAE buffer using known concentration DNA ladders, stained with ethidium bromide and visualized on UV transilluminator. The gels were documented using Gel Documentation System.
The PCR reactions were repeated many times to standardize the time and temperature of annealing. The PCR products were purified using Gel Extraction Kit (QIAGEN) according to manufacturer’s instructions and sequenced bi-directionally at Eurofins Genomics India Pvt. Ltd., Bangalore, India.

3.12.6 Phylogenetic analysis

The DNA sequence obtained for each candidate gene was searched for homology using BLAST against NCBI nucleotide database. The nucleotide sequence obtained for sos1, sos2, sos3, and nhx1 gene in mungbean cultivar PLM380 were aligned against the highly homologous sequences of the reference and other related genera retrieved from GenBank and the phylogenetic tree was constructed using MAFFT program.

3.12.7 Deduced amino acid sequence analysis

In order to study the sequence variation at protein level, the nucleotide sequence obtained in mungbean cultivar PLM380 for sos1, sos2, sos3, and nhx1 was translated into amino acid sequence using online translator tool and searched for similarity against the NCBI (GenBank) protein data base and for the presence of conserved domains online at NCBI. The deduced amino acid sequence of all genes obtained in mungbean cultivar (PLM380) were aligned with the already reported protein sequences of reference and other related legumes or modal plants available at Genebank database, NCBI to confirm the amplification of specific target sequence and analyzed for the extent of similarity using online ClustalW tool.

3.12.8 Sequence submission and assigned accession number

The nucleotide and amino-acid sequences obtained for the genes involved in salt overly sensitive (SOS) pathway and vacuolar antiporter in salt resistant mungbean genotype (PLM380) were deposited to the GenBank database at National Centre for Biological Informations (NCBI), to get the accession number.