# 5. Materials and Methods

## 5.1 Methodology adopted for genomic studies in barley seed development and seg8 mutant analysis

### 5.1.1 PLANT MATERIAL

Plants of *Hordeum vulgare* cv Barke, cv Bowman, the two-rowed barley spring cultivars, and the mutant *seg8* in Bowman background were grown in growth chambers at 20°C/18°C under 16 h light/8 h dark cycles. The developmental stage of the caryopses were determined in the mid-region of the ear as described by Weschke *et al.* (2000) and young developing seeds were harvested at 0, 2, 4, 6, 8, 10, 12 and 14 days after flowering (DAF). The developing caryopsis includes the maternal tissues, mostly pericarp and the filial tissues i.e. endosperm and embryo. The pericarp and embryo sac fraction were isolated by manual dissection. The tissue composition of the two fractions has been described (Sreenivasulu *et al.,* 2002).

### 5.1.2 EST IDENTIFICATION, ANNOTATION AND METABOLIC PATHWAY ASSIGNMENT

Based on the availability of resources generated at the Plant Genome Resource Centre, Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, 6,319 EST sequences from developing caryopses (Michalek *et al.,* 2002) were considered for annotation. Sequence similarity searches against databases were conducted with HUSAR (Heidelberg Unix Sequence Analysis Resource) using BlastX2 and BlastN2 from release 2.09 of the Blast2 (www.ncbi.nlm.nih.gov/; Altschul *et al.,* 1997). The BLASTX2 searches were performed by recent releases of the Swissprot and Gene Bank databases. By using a PERL script (IPK, Pleisner K) we extracted the top hits of BLAST score, E-value, percent identity, percent similarity and position of the alignment of both 5´ and 3´end sequences. We considered score value and alignment length from top hits to annotate ESTs into secure, putative and no assignment categories by length and score values (see Section 1.2.2 for criteria of categorization). Based on the putative gene identification by BLASTX results, we extracted EC numbers of all genes in the set belonging to metabolic pathways. For all the metabolic pathway analysis, we first collected information's from the plant data set of the KEGG database (http://www.genome.ad.jp/kegg/metabolism. Html) and based on EC number the classification of ESTs were performed and assigned to the respective pathways. To classify regulatory genes, the ESTs were categorized based on sequence homology to regulatory genes

of the KEGG database. After annotation and functional classification of developing caryopses barley ESTs, the starch biosynthetic pathway and glycolytic pathway ESTs were identified in our collection and expression data was extracted from macroarrays for all the clones belonging to the respective pathways. For an estimation of the number of unique genes represented on the array we used the results from a cluster analysis of a larger set of ESTs (Michalek et al. 2002) produced by using Stack Pack (Miller *et al.,* 1999).

*5.1.3 MACROARRAY PREPARATION*

**5.1.3.1 Selection of cDNA clones:** The cDNA clones (plasmid DNA) specific for developing barley caryopsis were obtained from the EST collection of the IPK plant genome resources centre (Michalek *et al.,* 2002). In the initial phase of this work 711 clones were used for array preparation. Clones were mostly selected from a cDNA library of developing caryopses (517 clones). A smaller number of clones were selected from a library of etiolated seedlings (73 clones) and roots (104 clones), giving a total of 691 and some additional fragments for internal control. In selecting the clones from the cDNA library of etiolated seedlings and roots, special emphasis was given to the carbohydrate metabolism and stress genes. In a second extended program, 1440 clones were used for macroarray preparation. Clones were selected preferentially from a cDNA library of developing caryopsis (1235 clones). Additionally, clones specific for etiolated seedlings (73 clones) and roots (104 clones), which were used in the 711 array were included, giving a total of 1412 and some additional internal control (same EST amplified independently or different EST clones coding for the same gene). All those sequences except a few of minor quality have been deposited in the EMBL sequence database and can also be obtained from a web-server at the IPK (http://www.pgrc.ipk-gatersleben.de).

**5.1.3.2 Amplification of cDNA inserts**: Inserts of cDNA clones (plasmid DNA) were amplified by PCR using slightly modified M13 universal (5´-CGACGTTGTAAAACGA CGGCCA) and reverse primers (5´-ACAGGAAACAGCTATGACCTTG) complementary to vector sequences flanking the cDNA inserts. For each cDNA clone, about 5 ng of plasmid DNA was used as template in a 50µl PCR reaction mix containing 10 mM Tris HCl [tris(hydroxymethyl)aminomethane] (pH 9.0), 50mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide, 1µM of each primer, and 0.6 units of Taq polymerase (Amplitaq Gold). Inserts were amplified (Master Cycler Gradient, Eppendorf, Hamburg, Germany) by using an amplification programme of 1 min denaturation at 94$^{o}$C and 30 cycles of 30 s at 94$^{o}$C,

followed by 30 s at 58°C and 2 min at 72°C. 5 µl of each reaction were separated on a 1.5 % agarose gel to evaluate the quality of amplicons. PCR reactions, which yield more than one fragment or resulted in low amounts of product, were repeated at slightly altered annealing temperature. After purification on QIA-quick columns according to the manufacturers protocol (Qiagen, Hilden, Germany) amplification products were again analysed on 1.5% agarose gels to estimate their DNA content and adjusted between 2.0 and 1.8.

**5.1.3.3 Spotting of cDNA fragments**: Concentration-adjusted PCR products were diluted 1:1 with 1 M NaOH, 5 M NaCl and deposited in duplicates on positively charged 5 x 9 cm nylon membranes (BiodyneB, Pall, Dreieich, Germany) by using a BioGrid robot equipped with spotting pins of 0.4 mm diameter (Biorobotics, Cambridge, UK). Three strokes with the spotting tool were used to transfer approximately 15 nl from each PCR product to the nylon membrane. After spotting was completed, the membranes were washed in 0.4 M NaOH, 1.5 M NaCl, neutralized in 0.5 M Tris, pH 7.5, 1.5 M NaCl and the DNA cross-linked to their surface by a brief UV treatment (120 mJ, Stratalinker, Stratagene, La Jolla, CA, USA). After washing in 2 x SSC arrays were dried for 1 h at 80°C and stored at room temperature until further use.

### 5.1.4 RNA EXTRACTION AND SYNTHESIS OF $^{33}$P-LABELLED cDNA PROBES

100 – 200 mg of the pericarp and embryo sac fraction were prepared from Barke, Bowman and s*eg8* caryopses by hand dissection and used to prepare total RNA as described by Heim *et al.* (1993). For synthesis of $^{33}$P-labelled cDNA, polyA$^+$-RNA was extracted from 35 µg of total RNA using oligo(dT)-magnetic beads (Dynal, Hamburg, Germany) according to the manufacturers recommendations. PolyA$^+$-RNA bound to the magnetic beads was used directly for synthesis of a covalently bound first strand cDNA library using Superscript reverse transcriptase (Life Technologies, Karlsruhe, Germany). $^{33}$P-labelled second strand cDNA free of unincorporated $^{33}$P-dCTP was obtained through a random priming reaction (Megaprime Labelling Kit, Amersham Pharmacia, Freiburg, Germany) with an increased amount of Klenow polymerase (10 units). After removal of the supernatant, $^{33}$P-labelled cDNA was eluted from the magnetic beads in 50 µl 2 mM EDTA by denaturation (3 min, 95°C) and filtered using a micro-centrifuge tube filter with a 0.2 µm Anapore membrane (Whatman, Göttingen, Germany).

### 5.1.5 PROCEDURE FOR cDNA MACROARRAY HYBRIDIZATION

Before the first hybridization experiment with labelled cDNA, every cDNA array was cycled through a mock hybridization including a probe removal procedure to detach loosely bound spotted DNA. After wetting the array in 2 x SSC, it was pre-hybridized for at least 3 h at 65°C in Church buffer (0.5 M sodium phosphate, pH 7.2, 7% (w/v) SDS, 1% (w/v) BSA, 1 mM EDTA) containing sheared salmon sperm DNA (10 μg/ml). Heat denatured (3 min, 95°C), labelled cDNA was added together with fresh hybridization buffer, and the filter was hybridized at 65°C for at least 12 h. cDNA arrays were washed three times with 40 mM sodium phosphate pH 7.2, 0.1% (w/v) SDS, 2 mM EDTA for 20 min at 65°C, wrapped in Saran wrap and exposed to an image plate of a Fuji BAS2000 phosphoimager (Fuji Photo Film Co., Tokyo, Japan) from 2 – 4 h. Labelled probes were completely removed from the filter by successive treatments with boiling washing solution, an alkaline treatment (0.4 M NaOH) for 15 min at 45°C and a neutralization reaction in 0.1 × SSC; 0.1 % (w/v) SDS; 0.2 M Tris HCl, pH 7.5. Successful removal of the labelled probe was controlled by long exposure (at least 4 h) of the array to an image plate.

### 5.1.6 ARRAY EVALUATION

The image data obtained from the phosphoimager were imported into the program package Array Vision (Imaging Research, St. Catharinas, Ontario, Canada) for spot detection and quantification of hybridization signals. Local background was subtracted from spot intensities and signal intensity of the duplicated spots for each cDNA fragment was averaged. In 711-macroarray, the signal intensities were normalized with respect to the total amount of radioactivity bound to the array and the signal intensities of the two spots representing each amplified cDNA fragment were averaged. To allow comparison of signal intensities across experiments in 1412-macroarray, the median of the logarithmically scaled intensity distribution for each experiment was set to zero (median centering of arrays, Eisen *et al.,* 1998). This procedure usually yields similar results like the previously used normalization (711 array) based on the total signal of an array, but improves the comparison of experiments with somewhat higher background values. Thereafter, the logarithmically scaled signal intensities of each gene was centered by its median across all experiments (median centering of genes, Eisen *et al.,* 1998), which emphasize the differential expression of genes irrespective of absolute signal intensities. These two procedures were repeated four more times and the resulting values were used for all clustering algorithms in J-Express (Disvik *et al.,* 2001). Since median centering of genes does not yield information about signal intensity,

we used the data after the first round of median centering of arrays to calculate non-logarithmic, normalized signal intensities. These normalized signal intensities were used to exclude genes with low signal intensities across all experiments for a more detailed analysis (see Results).

### 5.1.7 DATA FILTERING

For further analysis of the 1412 cDNA array analysis in "Barke" seed development, the complete dataset was reduced to cDNA fragments, which show differential expression across the experiments. Those cDNA fragments were selected based on two criteria. (1)To exclude cDNA fragments with signals always close to the background the normalized non-logarithmic signal intensity should exceed 3 arbitrary units (au) for at least one experiment. This value was compared with the normalized average background of 0.3 au and the maximal signal of 433 au. (2) The ratio between the minimal and the maximal signal for a cDNA fragment across all experiments had to exceed the factor of 10. The data set filtered in this way comprised 337 cDNA fragments, which fulfill both criteria.

### 5.1.8 CLUSTERING ALGORITHMICS

The goal of clustering is to group together genes (objects) with similar properties. J-Express provides a comprehensive set of clustering methods and informative visualizations. Hierarchical clustering and k-mean clustering (Eisen *et al*., 1998) tools of J-express software (Disvik *et al.,* 2001) were used for clustering the expression profiles.

**5.1.8.1 Usage of J-Express**: The normalized array data from different experiments were loaded as text files (tab delimited in a spreadsheet format), which allow selection of rows (expression values) and columns (gene identity) of every gene in a series of experiments. The feeded data file can be subjected to one of the analysis methods such as standard hierarchical and k-mean clustering. It represents a graphical user interface that integrates a number of viewing capabilities.

### 5.1.9 NORTHERN BLOTTING

Total RNA prepared from pericarp and embryo sac tissues of developing caryopses (0-8 DAF) harvested in two-day intervals was separated on a 1% agarose gel containing 15% formaldehyde and blotted overnight onto Hybond$N^+$ membranes (Amersham Pharmacia, Freiburg, Germany). Various fragments obtained by enzymatic digestions, which excluded

the polyA tail from the cDNAs, were used as probes after labelling with [$^{32}$P]dCTP. Membranes were sequentially hybridized with the following probes derived from cDNA clones: HY09N16 (*HvSUS2*) - a 312bp PCR-amplified sequence of the 3'-untranslated region (UTR); HY03B06 (putative transcription factor *FIL*) - a 412bp PstI-fragment; HW02F11 (vacuolar invertase) - a 283 PCR-amplified sequence of the 3'-UTR; HY09L21 (unknown gene called NucPro) - a 1.2kb DraI/PvuI fragment. Hybridizations were performed at 65°C, and the filters were washed at high stringency according to Church and Gilbert (1984). Signals on filters were quantified using a Fuji-BAS phosphoimager (Fuji Photo Film C., Tokyo, Japan). Loading of total RNA in each lane was monitored using a 26S rDNA probe (data not shown).

### 5.1.10 EXTRACTION AND DETERMINATION OF METABOLIC INTERMEDIATES

Frozen material (*seg8* and wild type caryopses) was extracted with trichloroacetic acid (Herbers *et al.,* 1997). Sugars including standards were determined by ion chromatography (DX-500, Dionex, USA) with pulsed amperometric detection. Separation was carried out on a Carbo-Pac PA1 column (4 x 250 mm) at 25 °C. Purest HPLC water and 0.3 M NaOH were used as eluents A ($NaH_2PO_4/Na_2HPO_4$, 1:1 molar ratio, 25 mM, pH 2.8) and B ($NaH_2PO_4/Na_2HPO_4$, 1:1 molar ratio, 125 mM, pH 2.9) respectively. The column was equilibrated with 3 % buffer A at a flow rate of 1 ml per minute. The gradient was produced by the following concentration changes: 15 min 25 % B, 18 min 35 % B, 24 min 83 % B, hold 83 % B for 6 min, return to 3 % B in 2 min. Nucleotides and nucleotide sugars were determined by ion chromatography (DX-500). Separation was carried out on a Vydac 302 IC column (4.6 x 250 mm) at 25 °C and the eluted sugars were photometrically detected at 260 nm. The column was equilibrated with buffer A at a flow rate of 2 ml per minute. The gradient was accomplished with buffer A ($NaH_2PO_4/Na_2HPO_4$, 1:1 molar ratio, 25 mM, pH 2.8) and buffer B ($NaH_2PO_4/Na_2HPO_4$, 1:1 molar ratio, 125 mM, pH 2.9). The gradient was produced by the following concentration changes: 2 min 0 % B, 9 min 11 % B, 18 min 100 % B, hold 100 % B for 2 min, return to 0 % B in 1 min.

### 5.1.11 DETERMINATION OF STARCH

Seeds from s*eg8* and wild type plants were freeze-dried and pulverized in a mortar-pessle. Soluble carbohydrates were extracted in 80% ethanol at 80°C for 1 h. After centrifugation, the supernatant was evaporated and dissolved in sterile water for enzymatic carbohydrate determination (Boeringer Mannheim, Germany). The remaining insoluble material was used

for starch determination after solubilising in 1 N KOH and hydrolysis with amyloglucosidase (Heim *et al.,* 1993).

## 5.2 Methodology adopted for salinity response studies in foxtail millet

### 5.2.1 PLANT MATERIAL AND SALINITY TREATMENTS

Seeds of foxtail millet (*Setaria italica* L.) cvs. Prasad (salt-tolerant) and Lepakshi (salt-sensitive) were procured from Andhra Pradesh Agricultural Experimental Station, Anantapur, India. Seeds were surface sterilized with 0.1% (w/v) sodium hypo chlorite solution for 5 min, thoroughly rinsed with distilled water and allowed to germinate on filter paper in petri dishes. Seeds were supplied with Hoagland medium supplemented with 0, 50, 100, 150, 200 and 250 mM NaCl. The Petri dishes were incubated at 25$^{o}$C under aseptic conditions for 5 days in the dark.

### 5.2.2 GROWTH MEASUREMENTS

The length of the primary shoot was measured for 100 seedlings (5-day-old) from each cultivar grown under control conditions (without NaCl) and different salt concentrations (50, 100, 150, 200 mM NaCl) in three independent experiments. The percentage of relative shoot growth inhibition was calculated from the mean shoot length measurements.

### 5.2.3 DETERMINATION OF SODIUM CONTENT

Sodium content was determined as described by Dionisio-Sese and Tobita, (1998). Dried seedlings (10 mg) were chopped into pieces of 5 mm length and placed in test tubes containing 20 ml distilled deionized water. The tubes were incubated in boiling water for 1 h and autoclaved at 121$^{o}$C for 20 min and cooled. The sodium content was determined by using an atomic absorption spectrophotometer (AA-660, Schimadzu; Tokyo, Japan).

### 5.2.4 ELECTROLYTE LEAKAGE

Electrolyte leakage was determined as described by Dionisio-Sese and Tobita, (1998). Fresh seedlings (200 mg) were cut into pieces of 5 mm length and placed in test tubes containing 10 ml distilled deionized water. The tubes were incubated in a water bath at 32$^{o}$C for 2 h and the initial electrical conductivity of the medium ($EC_1$) was measured. Afterwards, the samples were autoclaved at 121$^{o}$C for 20 min to release all electrolytes. Samples were then cooled to 25$^{o}$C and the final electrical conductivity ($EC_2$) was measured. The electrolyte leakage (EL) was calculated by using the formula $EL = EC_1/EC_2 \times 100$.

5.2.5 ESTIMATION OF MALONALDEHYDE (MDA) CONCENTRATION

The MDA content in 5-day-old seedlings was determined by the thio barbituric acid (TBA) reaction as described by Heath and Packer (1968). 100 mg fresh weight of seedlings were homogenised in 500µl of 0.1% (W/V) TCA. The homogenate was centrifuged at 9,000 x g for 5 min and 4 ml of 20% TCA containing 0.5% (W/V) TBA was added to 1 ml of the supernatant. The mixture was incubated at 95$^{o}$C for 30 min and then quickly chilled on ice. The contents were centrifuged at 9,000 x g for 15 min and the absorbance was measured at 532 nm in a Shimadzu 1601 spectrophotometer. The concentration of MDA was calculated using the MDA extinction coefficient (155 $mM^{-1}$ $cm^{-1}$).

5.2.6 cDNA ARRAYS

**5.2.6.1 Array design:** Barley EST's derived from cDNA libraries of developing caryopsis, roots and etiolated seedlings were used to create a cDNA array (Sreenivasulu *et al.,* 2002; materials and methods, section 5.1.3). Among the set of cDNA fragments, approximately 58 stress-related genes are included.

**5.2.6.2 Synthesis of $^{33}$P hybridization cDNA probe, hybridization and data normalization:** For synthesis of $^{33}$P-labelled cDNA probe, 35µg of total RNA was extracted from 5-day-old seedlings of control tolerant, 250 mM NaCl treated tolerant, control sensitive and 250 mM NaCl treated sensitive seedlings. Preparation of $^{33}$P-labelled second strand cDNA probes as well as the hybridization procedure has been described previously in the materials and methods (section 5.1.4 and 5.1.5). Pre-hybridization and hybridization was carried out at 60$^{o}$C. The cDNA arrays were washed three times with 40 mM sodium phosphate pH7.2, 1% SDS, 2mM EDTA for 30 min at 60$^{o}$C and exposed to the imaging plate of a Fuji BAS2000 phosphoimager (Fuji Photo Film, Tokyo, Japan) for 6 hr. The image data were imported into the program package Array vision (Imaging Research, St. Catharine's Ont., Canada) for spot detection and quantification of hybridization signals. The signal intensities were normalized with respect to the total amount of radioactivity bound to the array.

5.2.7 RT-PCR MEDIATED CLONING OF PHGPX cDNA

Total RNA from salt-tolerant and salt-sensitive seedlings treated with 250 mM NaCl was isolated as described by Heim *et al.,* (1993). RT-PCR was performed under standard conditions using an oligo d(T) primer for amplification of mRNA, followed by a PCR with two combinations of degenerated primers (forward: 5'-GTBAAYGTYGCHMARTGTG-3' / reverse 1: 5'-

TTRTCAAYMARRAAYTTRGWR AAG-3' , reverse 2: 5'-CRGCYTTRAADCKWGTGC-3'), which were designed on conserved regions of known PHGPX genes. A cDNA library from salt-treated seedlings of tolerant foxtail millet (5-day-old) was constructed using the ZAP express cDNA synthesis kit (Stratagene) and screened under low stringency conditions using the $\alpha^{32}$P-labelled cDNA fragment (316 bp) obtained by RT-PCR.

5.2.8 SOUTHERN HYBRIDIZATION

Genomic DNA from seedlings was isolated, 10 µg of DNA was digested with BamHI, EcoRI, HindIII, PvuII and XhoI and separated on a 1% agarose gel. The gel was blotted overnight onto a Hybond-$N^+$ nylon membrane (Amersham Braunschweig, Germany). The 316 bp RT-PCR PHGPX fragment of the millet was used as probe after labelling with [$\alpha$-$^{32}$P]dCTP. Hybridizations were performed at 65°C, and the filter was washed twice with 2xSSPE / 0.1% SDS, twice with 1xSSC/ 0.1% SDS, and once with 0.5xSSC/0.1% SDS at 65°C for 15 min each.

5.2.9 NORTHERN BLOT ANALYSIS

RNA isolation and gel blot analysis were performed as described in the materials and methods (section 5.1.9). Filters were hybridized to the $^{32}$P-labelled PCR fragment of the PHGPX gene as probe at 55$^o$C according to the method of Church and Gilbert, (1984). The hybridization signals were quantified using the Bio Image Analyser BAS 2000 (Fuji Photo Film Co., Tokyo, Japan). To check the amount of total RNA loaded on each lane, filters were rehybridized with a $^{32}$P-labelled 26S rRNA fragment of potato.

5.2.10 CHARACTERIZATION OF THE SALT-INDUCED 25kD PROTEIN

**5.2.10.1 Protein extraction and estimation of protein content:** Five-day-old seedlings (100 mg) of both cultivars grown at 250 mM NaCl and control conditions were ground to a fine powder with liquid nitrogen and homogenized with ice-cold 50 mM Tris-HCl buffer (pH 7.4). The extracts were centrifuged for 20 min at 8,000 g at 4$^o$C. Protein concentration was determined by the Dye-binding assay (Bradford, 1976) using bovine serum albumin (BSA) as standard.

**5.2.10.2 SDS-PAGE analysis:** An equal amount of each protein sample (35 µg) was loaded on 12-15% gradient SDS-PAGE gel. After running, the gels were stained with silver nitrate solution for protein detection (Laemmli, 1970).

**5.2.10.3 Purification of 25 kD protein:** Five-day-old seedlings (5 g) of the tolerant cultivar grown at 250 mM NaCl were homogenized in liquid nitrogen and extracted with 50 mM Tris-HCl buffer (pH 7.4), containing 1 mM ethylenediamine tetra-acetic acid (EDTA), 2mM phenylmethylsulphonyl fluoride (PMSF), 0.6% insoluble polyvinylpyrrolidone (PVP) and 0.5 M sucrose. The homogenate was filtered through a nylon sieve (40 μm) and centrifuged at 8,000 g for 10 min. A DEAE-Sepharose (Sigma) column (bed: 10 x 2.5 cm) was pre-equilibrated with Tris-HCl buffer (50 mM pH 7.2) and the supernatant containing proteins were subjected for ion-exchange chromatography. The sample was eluted through a NaCl step-gradient (0.2, 0.4, 0.6, 0.8 and 1.0 M) and 1 ml fractions were collected. The absorbance of each fraction was monitored at 280 nm and total peroxidase activity was determined. The fractions number 12-18 containing peroxidases were pooled, lyophilised and subjected to FPLC (Superose) to achieve higher purity. The column was pre-equilibrated and eluted with PBS buffer (pH 7.4) as flow-through. The salt-induced 25 kD protein defined by the major peak was collected and purified to the maximum extent.

**5.2.10.4 Amino acid sequencing:** The purified protein (salt-induced 25 kD protein) was electrophorated on a 12% SDS-PAGE (Laemmli, 1970) and electroblotted onto polyvinylidene difluoride (PVDF) membrane (Millipore) using the Multiphor II (LKB, Pharmacia) electrophoretic transfer apparatus according to the manufacturer's protocol. 10 mM 3-[cyclohexylamino]-1-propane-sulphonic acid (CAPS; pH 11) was used as transfer buffer. The membrane was stained by coomassie blue, and that part of the membrane containing the purified protein was subjected to the sequencer LF3400 (Beckman Instruments Fullerton, Ca, USA).