

Preface

The phenomenon of seed development remains an intriguing problem and much effort has been directed toward its understanding. In this thesis, particular attention is drawn to early seed development and the process that lead up to the phase of storage product accumulation in barley by an integrative approach including genomics, biochemistry, physiology and histology. In addition, close inspection of early seed development in the *seg8* mutant of barley, which is defective in endosperm development, is carried out by genomic studies to understand the importance and complexity of maternal and filial tissue interactions during early caryopses development. By using the EST-array resources generated in this project we conducted gene expression analysis in foxtail millet cultivars differing in salt sensitivity by cross-hybridization experiments. The foxtail millet experimental system has been chosen because of the availability of two different genotypes differing in their sensitivity to salinity, which is absent in barley.

The thesis is comprised of four chapters. Chapter-1 is dealing with a general introduction of the main technology used; macroarrays based on expressed sequence tags (ESTs). A results and discussion section deals with construction and employment of an EST-based macroarray and its quality control. Chapter-2 consists of early seed development studies in the cultivar Barke of barley. Chapter-3 deals with early seed development in the *seg8* mutant of barley and chapter-4 with gene expression during salinity stress in foxtail millet. Each chapter (2, 3 and 4) starts with an introduction into the specific research area, describes the obtained results and discusses the relevant problems. To avoid repetitions Materials and Methods adopted are described in one section at the end of the thesis.

Contents

0 ABSTRACT.....	1
0 ZUSAMMENFASSUNG.....	4
CHAPTER 1: Expressed Sequence Tags (ESTs) and cDNA arrays as tools for global gene expression analysis in barley.....	8
1.1 AN INTRODUCTION TO EXPRESSED SEQUENCE TAGS (ESTs).....	8
1.1.1 EST-based gene discovery - its merits and inherent limitations.....	9
1.1.1.1 <i>cDNA library generation</i>	10
1.1.1.2 <i>EST sequencing and quality check</i>	10
1.1.1.3 <i>EST clustering/Gene content</i>	11
1.1.1.4 <i>Employing bioinformatic tools for annotation of ESTs</i>	11
1.1.2 High throughput transcript profiling by EST arrays.....	13
1.1.2.1 <i>Data mining</i>	14
1.1.2.2 <i>Array development</i>	15
1.1.2.3 <i>Probe synthesis/hybridization</i>	16
1.1.2.4 <i>Data analysis</i>	16
1.1.3 Biological interpretation of expression data.....	18
1.2 RESULTS AND DISCUSSION.....	19
1.2.1 EST generation from developing caryopsis library (0-15 DAF)	19
1.2.2 Annotation and functional classification of barley ESTs from developing caryopses....	19
1.2.3 Preparation of an EST macroarray	22
1.2.4 Performance of an EST macroarray containing 711 clones.....	23
1.2.5 Performance of an EST macroarray containing 1412 clones.....	26
1.2.6 Expression analysis of selected genes.....	27
CHAPTER 2: A Genomic approach to barley seed development.....	31
2.1 INTRODUCTION.....	31
2.1.1 Aspects of seed development.....	31
2.1.2 Molecular physiology of caryopses development during early stage.....	33
2.1.3 Carbohydrate metabolism and its role in seed development.....	34
2.1.3.1 <i>Role of sucrose / hexose transporters in seed development</i>	34
2.1.3.2 <i>Catabolism of sucrose to hexoses during seed development</i>	35
2.1.3.3 <i>Sugar sensing mechanisms during seed development</i>	36
2.1.3.4 <i>Sugar-regulated genes during seed/plant development</i>	36
2.1.4 Genomic approaches in seed development.....	37
2.2 RESULTS.....	39
2.2.1 Barley seed development: seed morphology and tissue preparation.....	39
2.2.2 Identification of 16 clusters representing tissue – and development-specific expression profiles during early caryopses development.....	40

2.2.3 Identification of functional classes of genes expressed specifically in pericarp tissue during caryopses development.....	43
2.2.4 Identification of functional classes of genes expressed in filial tissue during pre-storage and initial storage phase of developing caryopses.....	47
2.2.5 Carbohydrate metabolism during seed development	55
2.2.5.1 <i>Expression patterns of starch metabolic pathway genes in maternal and filial tissues..</i>	55
2.2.5.2 <i>Expression patterns of glycolysis metabolic pathway genes in maternal and filial tissues</i>	58
2.3 DISCUSSION.....	60
2.3.1 Pericarp specific expression during the development of barley caryopses (0-12 DAF)...	61
2.3.2 Gene expression map of the filial tissue during caryopses development (0-12 DAF).....	62
2.3.3 Interaction of maternal and filial tissues with reference to starch storage function	64
2.4 SUMMARY.....	66
CHAPTER 3: <i>seg8</i> mutant analysis during seed development	67
3.1 INTRODUCTION.....	67
3.2 RESULTS.....	70
3.2.1 Fresh weight of developing caryopses of <i>seg8</i> and wild type.....	70
3.2.2 Starch content in caryopses of <i>seg8</i> and wild type.....	71
3.2.3 Characteristic changes in sugar and metabolite concentrations in <i>seg8</i> and Bowman during pre-storage and storage phase in pericarp and embryo sac fractions.....	71
3.2.4 Anatomy and starch distribution pattern in developing grains of <i>seg8</i> mutant and wild type.....	73
3.2.5 Characteristic changes of gene expression in developing caryopses of <i>seg8</i> and Bowman	74
3.2.6 Different expression profiles of genes encoding enzymes of the sugar-to-starch pathway monitored in maternal and filial fractions of developing <i>seg8</i> mutant and Bowman wild type grains.....	76
3.2.7 mRNA expression of some transporter genes is drastically reduced in the filial fraction of developing <i>seg8</i> grains, as compared to the wild type.....	78
3.3 DISCUSSION.....	79
3.3.1 The <i>seg8</i> genomic environment integrated in "Bowman" displays the same features described for the original mutant identified in "Klages".....	79
3.3.2 Low mRNA expression of genes encoding key enzymes in the starch biosynthesis pathway may cause the reduced starch content of the mutant grain.....	79
3.3.3 High sucrose levels in the maternal and filial fraction during storage phase hint to a delay in sugar utilization and reduced starch accumulation in mutant's endosperm.....	80
3.3.4 A defect in starch accumulation can be expected for the developing gynoeceium.....	81
3.4 SUMMARY	81

CHAPTER 4: Expression analysis of foxtail millet genotypes differing in salt tolerance.	82
4.1 INTRODUCTION.....	82
4.2 RESULTS.....	87
4.2.1 Growth attributes.....	87
4.2.2 Sodium content measurements.....	87
4.2.3 Effect of salinity on electrolyte leakage.....	88
4.2.4 Effect of salinity on malonaldehyde content.....	89
4.2.5 High-throughput expression analysis of salt stress responsive genes.....	89
4.2.6 Hydrogen peroxide scavenging enzymes.....	91
4.2.7 Isolation and identification of a full-length cDNA coding for a PHGPX from millet.....	91
4.2.8 Identification of PHGPX gene family in millet.....	93
4.2.9 Salt-specific induction of the PHGPX protein (25 kD).....	94
4.2.10 Purification of the salt-induced 25 kD PHGPX protein.....	95
4.2.11 Amino acid sequence analysis of salt-induced 25 kD protein.....	96
4.3 DISCUSSION.....	96
4.3.1 Application of barley macroarrays to foxtail millet.....	96
4.3.2 Differential response of physiological parameters to salinity stress in salt-tolerant and salt sensitive seedlings of foxtail millet.....	97
4.3.3 The possible role of hydrogen peroxide scavenging enzymes in salt-mediated oxidative stress tolerance.....	97
4.4 SUMMARY.....	100
5. MATERIALS AND METHODS SECTION.....	101
5.1 Methodology adopted for genomic studies in barley seed development and <i>seg8</i> mutant analysis.....	101
5.1.1 Plant material.....	101
5.1.2 EST identification, annotation and metabolic pathway assignment.....	101
5.1.3 Macroarray preparation.....	102
5.1.4 RNA extraction and synthesis of ³³ P-labelled cDNA probes.....	103
5.1.5 Procedure for cDNA macroarray hybridization.....	104
5.1.6 Array evaluation.....	104
5.1.7 Data filtering.....	105
5.1.8 Clustering algorithmic.....	105
5.1.9 Northern blotting.....	105
5.1.10 Extraction and determination of metabolic intermediates.....	106
5.1.11 Determination of starch.....	106
5.2 Methodology adopted for salinity response studies in foxtail millet.....	107
5.2.1 Plant material and salinity treatments.....	107
5.2.2 Growth parameters.....	107
5.2.3 Determination of sodium content.....	107
5.2.4 Electrolyte leakage.....	107
5.2.5 Estimation of malonaldehyde (MDA) concentration.....	108
5.2.6 cDNA arrays.....	108

5.2.6.1 Array design.....	108
5.2.6.2 Synthesis of ³³ P hybridization cDNA probe, hybridization and data normalization	108
5.2.7 RT-PCR mediated cloning of PHGPX cDNA.....	109
5.2.8 Southern hybridization.....	109
5.2.9 Northern blot analysis.....	109
5.2.10 Characterization of the salt-induced 25 kD protein.....	109
5.2.10.1 Protein extraction and estimation of protein content.....	109
5.2.10.2 SDS-PAGE analysis.....	110
5.2.10.3 Purification of 25 kD protein.....	110
5.2.10.4 Amino acid sequencing.....	110
6. REFERENCES.....	111
7. ACKNOWLEDGEMENTS.....	123

Index of Tables

Table 1: Expressed sequence tags of major cereals in dbEST.....	9
Table 2: Web sites useful for EST annotation.....	13
Table 3: Design principles of arrays used for expression analysis.....	16
Table 4: Analytical tools with application to gene expression and worldwide web addresses of softwares for array data analysis from the public domain as well as the private sector	17
Table 5 cDNA clones that are preferentially expressed in pericarp.....	25
Table 6 cDNA clones preferentially expressed in the embryo sac.....	26
Table 7 Members of cluster 1_1, 1_2 and 1_3 showing higher expression in pericarp tissue....	45
Table 8 Members of cluster 2_1, 2_2 and 2_3 showing higher expression in the filial tissue during the pre-storage phage.....	48
Table 9 ESTs included in cluster 3_2 showing up regulation of expression in the intermediate phase of development in filial tissues.....	50
Table 10. ESTs included in cluster 4_1, 4_2, 4_3 and 4_4 showing up regulation of expression in the storage phase of the filial tissues.....	52
Table 11 Members of cluster 5 showing high expression especially in the storage phase of the filial tissues.....	55
Table 12: ESTs belongs to the sugar to starch pathway are preferentially down regulated in developing caryopses of <i>seg8</i> mutant.....	75
Table 13 cDNA clones that are preferentially expressed in salt-treated tolerant seedlings.....	90

Index of Figures

Fig. 1 A diagrammatic representation of EST-array technique.....	14
Fig. 2 Scatter plot representation of EST annotation data.....	20
Fig. 3a Annotation of 1400 ESTs from developing caryopses (0-12 DAF).....	21
Fig. 3b Functional classification of ESTs from developing caryopses (0 to 12 DAF).....	21
Fig. 4 Segment of a cDNA macroarray.....	23
Fig. 5 Comparison of the normalized signal intensities obtained from two independently spotted arrays hybridized with the same labelled cDNA (A) and from one array hybridized successively with labelled cDNA from embryo sac and pericarp tissues of the developing barley grain 1-7 DAF (B).....	24
Fig. 6 Levels of transcripts differentially accumulated in pericarp and embryo sac of developing caryopses measured by northern analysis	29
Fig. 7 Comparison of expression levels of selected genes resulting from cDNA array and Northern blot analyses.....	29
Fig. 8 Schematic representation of the histological organization of a barley caryopsis.....	35
Fig. 9 Developing caryopses and hand-dissected maternal (pericarp) and the filial (endosperm and embryo) fractions.....	40
Fig.10 Tissue and development-specific expression profiles identified by k-mean cluster analysis.....	42
Fig. 11 Groups of genes specifically expressed in the maternal tissue (0-12 DAF).....	44
Fig. 12 Up regulation of gene expression (cluster 1_2 and 1_3) in the maternal fraction demonstrated by the Eisen method.....	44
Fig. 13 Groups of genes specifically expressed in filial tissues (0 to 12 DAF).....	47
Fig. 14 Expression of photosynthetic genes in the filial fraction of the caryopsis during 4 to 8 DAF.....	51
Fig. 15 Schematic representation of the sucrose-starch pathway and mRNA level of the respective enzymes as determined by expression analysis in both maternal pericarp (p) and filial tissue containing endosperm and embryo (e).....	57
Fig. 16 Schematic representation of the glycolysis pathway and mRNA level of the respective enzymes as determined by expression analysis in both the maternal fraction containing mainly pericarp (p) and filial tissues containing endosperm and embryo (e).....	59

Fig. 17 After separation of maternal (pericarp) from the filial part (embryo sac), fresh weight of both fractions was estimated and the fresh weight ratio was calculated. On X-axis the developmental scale 2-14 DAF (Days After Flowering) is given in every two day intervals...	70
Fig. 18 Starch content in caryopses of <i>seg8</i> and wild type.....	71
Fig. 19 Sugar and metabolite measurements determined in maternal and filial fraction of <i>seg8</i> and wild type of developing caryopses.....	72
Fig. 20 Starch distribution pattern of <i>seg8</i> caryopses shown in median-transversal sections (8-12 DAF) by Iodine staining.....	73
Fig. 21 Comparison of the normalized signal intensities obtained from two independent experiments (experiment 1 and 2).....	74
Fig. 22 Expression data of EST clones with homology to genes coding sugar to starch pathway were selected.....	77
Fig. 23 Expression profiles of transporter genes in filial fraction of mutant (a) and wild type (b) during early and mid caryopses development.....	78
Fig. 24 A proposed model for pathways leading to the induction of reactive oxygen species (superoxide radical, hydrogen peroxide and hydroperoxides) during NaCl treatment and the role of the protective antioxidative enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX) and phospho glutathione peroxidase (PHGPX) in scavenging superoxide, hydrogen peroxide and hydroperoxide radicals respectively.....	84
Fig. 25 Differences in root and shoot length of 5-day old seedlings of a salt-tolerant (P – Prasad) and a salt-sensitive (L – Lepakshi) foxtail millet cultivar grown under control conditions (CP – control Prasad; CL – control Lepakshi) and at different NaCl concentrations (SP – salt-treated Prasad; SL – salt-treated Lepakshi).....	87
Fig. 26 Na ⁺ accumulation in 5-day-old seedlings of the tolerant and sensitive foxtail millet cultivar grown at different NaCl concentrations.....	88
Fig. 27 Electrolyte leakage rate measured in cells of 5-day-old seedlings of the tolerant and sensitive foxtail millet cultivars grown at different salt concentrations.....	88
Fig. 28 Variation in the MDA content of salt-tolerant and salt-sensitive seedlings of foxtail millet grown under different concentrations of NaCl.....	89
Fig. 29 Northern blot analysis of PHGPX mRNA accumulation following salt treatment (250 mM NaCl) in the tolerant foxtail millet cultivar as compared to the salt-sensitive cultivar.....	91
Fig. 30 Amino acid sequence alignment of cDNA clone isolated from <i>Setaria italica</i> PHGPX (SiGPX) with PHGPX sequences from other species.....	92
Fig. 31 Southern blot analysis of <i>Setaria italica</i> PHGPX gene.....	94

Fig. 32 Protein patterns of 5-day-old tolerant seedling samples grown under control (Ct) conditions and different types of stress such as 150 mM NaCl (S), drought (D), high temperature (H) and cold (C) depicted on a 12-15% gradient acrylamide gel..... 94

Fig. 33 Purification of the salt-induced 25 kD protein from NaCl-treated tolerant seedlings by DEAE-Sepharose and FPLC..... 95